Investigating the Anti-Viral Property of Verotoxin and its Receptor Gb₃ in Preventing Primary HIV-1 Infection

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Biochemistry
University of Toronto

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2011

Abstract

Verotoxin produced by Enterohemorrhagic *E. coli* is comprised of a catalytic A subunit and a receptor Gb$_3$ binding B subunit pentamer. VT causes protein synthesis inhibition by ribosomal inactivation in Gb$_3$ positive cells via receptor mediated endocytosis and retrograde transport to the ER. We propose that verotoxin is a novel inhibitor for HIV-1 infection. Experiments conducted using VT treated Jurkat-C T cells and PHA/IL-2 activated human PBMCs reveal the anti-HIV-1 property of VT is receptor Gb$_3$ independent since the catalytic A subunit alone is sufficient for inhibition. Possible mechanism of action involves mild inhibition of protein synthesis and cell proliferation. Recent findings in our lab suggest Gb$_3$ is a natural resistance factor for HIV-1 infection, which was further investigated by selecting a Gb$_3$ low subpopulation in THP-1 cells using VT treatment. Selected THP-1 cells were completely resistant to HIV-1 infection, however decreased surface CXCR4 expression may be a cause.
Acknowledgments

I would like to thank my supervisor Dr. Cliff Lingwood for giving me this wonderful opportunity to pursue HIV-1 research in the lab. Also thank my committee members Dr. Don Branch and Dr. Craig Smibert for their inputs.

I would also like to thank all the past and present members of the Lingwood lab and Branch lab for all the help they have given me during my research. Especially to Beth, who have helped me tremendously in designing many of the experiments and given me advices in general research. Thanks to Darinka for training me inside the Level 3 lab and preparing the virus used in this study.

Finally, I would like to thank my parents for their continual support throughout the whole process. Most importantly, I would like to thank Scott. For without your emotional support this would never be possible.
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<tbody>
<tr>
<td>α4Gal transferase</td>
<td>Gb₃ synthase</td>
</tr>
<tr>
<td>α-GAL</td>
<td>α-galactosidase A</td>
</tr>
<tr>
<td>AdaGb3</td>
<td>Adamantyl globotriosylceramide</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCR5</td>
<td>CC-chemokine receptor 5</td>
</tr>
<tr>
<td>CXCR4</td>
<td>CXC-chemokine receptor 4</td>
</tr>
<tr>
<td>d.selTHP-1</td>
<td>Double VT1 selected THP-1 cells</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorting</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Gag</td>
<td>Group specific antigen</td>
</tr>
<tr>
<td>GalCer</td>
<td>Galactosylceramide</td>
</tr>
<tr>
<td>Gb₃</td>
<td>Globotriaosylceramide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Gb₄</td>
<td>Globotetraosylceramide</td>
</tr>
<tr>
<td>GlcCer</td>
<td>Glucosylceramide</td>
</tr>
<tr>
<td>Gp</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>GSL</td>
<td>Glyosphingolipid</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HUS</td>
<td>Hemolytic uremic syndrome</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>LacCer, LC</td>
<td>Lactosylceramide</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>m.o.i.</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside reverse transcriptase inhibitor</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylimic gel electrophoresis</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
</tbody>
</table>
PIC  Pre-integration complex
PI  Protease inhibitor
PMA  Phorbol myristate acetate
PR  Protease
RNA  Ribonucleic acid
RT  Reverse transcriptase
RT  Room temperature
SAHA  Suberoylanilide hydroxamic acid
selTHP-1  VT1 selected THP-1 cells
SDS  Sodium dodecyl sulfate
TBS  Tris buffered Saline
TLC  Thin layer chromatography
TM  Transmembrane protein
VT  Verotoxin
WHO  World health organization
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1 Chapter 1: Introduction and Background

1.1 HIV-1/AIDS

1.1.1 AIDS the pandemic

Acquired Immunodeficiency Syndrome (AIDS) is one of the most prevalent worldwide infectious diseases. In 2009, a global report on this pandemic published by the World Health Organization estimated there are a total of 33.3 million people living with HIV (UNAIDS/WHO). In that year alone, 2.9 million people were newly infected. Over 7000 new infections occur each day, 97% of which are in developing countries. The most severely affected region is in Sub-Saharan Africa, with a total of 22.5 million, followed by South and South-East Asia with a total of 4.1 million. In 2009 alone, over 1.8 million people have died of AIDS and 260,000 of these were children under the age of 15. Importantly, the number of new infections each year is currently declining since the peak year of 1997 due to global efforts in disease prevention and treatment. However, women continue to be at higher risk to HIV-1 compared to men as a result of social inequality. About 60% of the infected population in Sub-Saharan Africa is female. The most common way for women to become infected is through unprotected sex. The ABC program, which promotes “Abstinence, Be faithful and use Condoms” has been implemented in many African countries with moderate success (S. Cohen, 2004). However, cultural situations often do not encourage this approach and may not be feasible for women who are dependent on their male partners for financial support which force them to be in high risk relationships.

1.1.2 Discovery of AIDS/HIV-1

The first cases of AIDS were reported in 1981 by the US Center for Disease Control and Prevention, where a group of gay men were found to be infected by a strain of pneumonia susceptible only to immune compromised individuals (Gottlieb, 2006). A month later, another group of gay men was found to have developed the rare Kaposi’s sarcoma, also indicative of a weakened immune system (Centers for Disease Control and Prevention, 1981). In 1983, the
causative agent for this disease was isolated by the group at Pasteur Institute in Paris, France (Wain-Hobson et al., 1991), which was then named the HIV-1 virus in 1986 (Coffin et al., 1986).

1.1.3 HIV-1 classification and structure

HIV-1 is classified as a Lentivirus and belongs to the family of Retroviridae. It is capable of infecting many species and results in extended duration of illness with a long incubation time between the initial exposure to the virus and the appearance of the symptoms (Levy, 1993). There are three major groups of HIV-1: M, N and O, and a tentative P group. Nine subtypes or clades have been indentified: A-D, J-H and J-K. The majority of infections occur with group M viruses of B and C clades. The difference between groups or clades of virus exists primarily in their envelope gene sequence. HIV-1 is transmitted as single-stranded RNA virus enclosed within an envelope (Figure 1.1). The outer envelope consists of a lipid-bilayer with phospholipids taken from the host cell membrane during budding of the virus. Embedded in this lipid-bilayer is the viral Env protein responsible for host cell recognition and fusion entry. Env consists of a trimeric glycoprotein, gp120, which masks a transmembrane gp41 stalk (Center et al., 2002; Chan et al., 1997). A proteinous matrix made up of viral protein p17 lies just underneath the lipid membrane and provides structural support for the virus. The inner most layer is the conical viral core composed of a capsid protein, p24, which contains two copies of plus (+) sense single-stranded viral genomic RNA of ~9.2 kilo bases with its associated proteins including reverse transcriptase, integrase and proteases.
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<table>
<thead>
<tr>
<th>Name</th>
<th>Size</th>
<th>Function</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gag MA</td>
<td>p17</td>
<td>membrane anchoring; env interaction; nuclear</td>
<td>virion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>transport of viral core (myristylated protein)</td>
<td></td>
</tr>
<tr>
<td>CA</td>
<td>p24</td>
<td>core capsid</td>
<td>virion</td>
</tr>
<tr>
<td>NC</td>
<td>p7</td>
<td>nucleocapsid, binds RNA</td>
<td>virion</td>
</tr>
<tr>
<td></td>
<td>p6</td>
<td>binds Vpr</td>
<td>virion</td>
</tr>
<tr>
<td>Pol Protease (PR)</td>
<td>p15</td>
<td>Gag/Pol cleavage and maturation</td>
<td>virion</td>
</tr>
<tr>
<td>Reverse Transcriptase (RT)</td>
<td>p66, p51</td>
<td>reverse transcription, RNase H activity</td>
<td>virion</td>
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<tr>
<td>RNase H</td>
<td>p15</td>
<td>DNA provirus integration</td>
<td>virion</td>
</tr>
<tr>
<td>Integrase (IN)</td>
<td>p31</td>
<td></td>
<td>virion</td>
</tr>
<tr>
<td>Env gp120/gp41</td>
<td></td>
<td>external viral glycoproteins bind to CD4 and</td>
<td>plasma membrane, virion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>secondary receptors</td>
<td>envelope</td>
</tr>
<tr>
<td>Tat</td>
<td>p16/p14</td>
<td>viral transcriptional transactivator</td>
<td>primarily in nucleolus/nucleus</td>
</tr>
<tr>
<td>Rev</td>
<td>p19</td>
<td>RNA transport, stability and utilization factor (phosphoprotein)</td>
<td>primarily in nucleolus/nucleus shuffling between nucleolus and cytoplasm</td>
</tr>
<tr>
<td>Vif</td>
<td>p23</td>
<td>promotes virion maturation and infectivity</td>
<td>cytoplasm (cytosol, membranes), virion</td>
</tr>
<tr>
<td>Vpr</td>
<td>p10-15</td>
<td>promotes nuclear localization of preintegration complex, inhibits cell division, arrests infected cells at G2/M</td>
<td>virion nucleus (nuclear membrane?)</td>
</tr>
<tr>
<td>Vpu</td>
<td>p16</td>
<td>promotes extracellular release of viral particles; degrades CD4 in the ER; (phosphoprotein only in HIV-1 and SIVcpz)</td>
<td>integral membrane protein</td>
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<tr>
<td>Nef</td>
<td>p27-p25</td>
<td>CD4 and class I downregulation (myristylated protein)</td>
<td>plasma membrane, cytoplasm, (virion?)</td>
</tr>
<tr>
<td>Vpx</td>
<td>p12-16</td>
<td>Vpr homolog present in HIV-2 and some SIVs, absent in HIV-1</td>
<td>virion (nucleus?)</td>
</tr>
<tr>
<td>Tev</td>
<td>p28</td>
<td>tripartite tat-env-rev protein (also named Tnv)</td>
<td>primarily in nucleolus/nucleus</td>
</tr>
</tbody>
</table>

1.1.4 HIV-1 infection and life cycle

HIV-1 is transmitted by direct contact with contaminated bodily fluids such as blood, semen, vaginal fluid and breast milk. The most common routes of transmission include unprotected sexual intercourse, injection of contaminated blood, needle sharing between drug users and mother to child transmission during the prenatal period or through breast feeding (Jaffe et al., 1983).

HIV-1 infection is initiated when gp120 recognizes its host cell primary receptor, CD4 (Dalgleish et al., 1984). CD4 is mainly expressed on a subset of T cells and to a lesser extent on
monocytes and macrophages. The normal function of CD4 is to interact with major histocompatibility complex II on antigen presenting cells to induce an immune response to foreign antigens (Gay et al., 1988). The interaction of gp120 and CD4 is very weak and some HIV-1 permissible cells express very little CD4 (Ugolini et al., 1999). Therefore other factors are involved. The binding to CD4 triggers conformational changes in gp120 that result in the recruitment of seven-transmembrane G-protein coupled chemokine co-receptors. The specificity of co-receptor used is determined by the sequence of the variable region V3 loop on gp120 (Speck et al., 1997). The binding of CD4, gp120 and co-receptor results in further conformational changes that expose the transmembrane gp41. gp41 by insertion into the host cell membrane initiates viral-host cell membrane fusion. The p17 matrix protein is then dissolved intracellularly and the viral preintegration complex (PIC) is formed within the cytosol. The PIC consists of genomic RNA and associated viral proteins, including p17, integrase and Vpr. The PIC is then transported to the nucleus where it can form 2-LTR circular DNA and/or be integrated in the host genome.

Immediately following viral entry, the viral genomic RNA is converted into double stranded DNA by reverse transcriptase and ribonuclease H (Varmus, 1988). The dsDNA is then actively transported via the PIC into the nucleus which is independent of host cell division (Bukrinsky et al., 1992). The insertion of viral dsDNA into the host genome is catalyzed by integrase and results in the formation of a provirus. The integration sites correlate strongly with regions enriched in genes that are transcriptionally active (Brady et al., 2009). The provirus can remain dormant for many years. Provirus can be activated for expression by a number of factors, which are both viral and cellular in nature (Cullen, 1991; Reddy & Dasgupta, 1992). The first genes expressed are non-structural regulatory factors which control the course of viral production (Rosenberg & Fauci, 1991). Tat is required for the initiation of RNA transcription and production of full length RNA. The transcribed RNA is then multiply spliced to form mature RNA. The transport of mature RNA out of the nucleus is regulated by Rev. Viral protein is then synthesized using the protein synthesis machinery of the host cell. Newly made viral proteins assemble at the host cell surface and an immature virus is formed by budding off part of the host cell plasma membrane. Maturation of the HIV-1 virus is achieved by protease processing to release individual mature viral proteins. The now mature HIV-1 can begin another round of infection. Figure 1.3 summarizes the HIV-1 infection cycle.
Figure 1.3 – HIV-1 infection cycle. (Adapted from http://www.webbooks.com/eLibrary/ON/B0/B22/05MHIV-1.html)
1.1.5 HIV-1 co-receptors

There are two major co-receptors used by the HIV-1 virus in conjunction with its primary receptor, CD4, to infect target cells. They are the chemokine receptor CCR5 and CXCR4. HIV-1 initial exposure and transmission is carried out by CCR5 by so-called R5 tropic viruses, whereas CXCR4 is used by so-called X4 tropic virus and is primarily involved in the more advanced stages of HIV-1/AIDS. This is because the initial entry site of HIV-1, the epithelial cell lining of the gastrointestinal system expresses CCR5 rather than CXCR4 (Meng et al., 2002), which allows selective transcytosis of R5 virus across the epithelial lining. Also R5 tropic virus preferably infects monocytes/macrophages which are present in large numbers in local lymphatic tissues. Furthermore, CD4+ T cells can express CCR5 and depletion of CCR5 +/CD4 + T cells in the GI-associated lymphoid tissue (GALT) is a prominent finding in all stages of HIV disease, loss of these GALT CD4+ T cells being more than when comparing to CD4 + T cells in blood and lymph nodes (Brenchley et al., 2004). On the other hand, X4 tropic viruses primarily infect CXCR4-expressing T cells in the blood and lymph nodes. These receptors belong to the superfamily of seven-transmembrane G-protein coupled receptors that in the absence of HIV-1 are responsible for a number of cellular functions including development, angiogenesis, trafficking and immune response.

There are several CC chemokines that have been identified to bind CCR5. Among those, CCL3, CCL4, CCL5 and CCL8 were shown to have suppressive properties in HIV-1 infection (Blanpain et al., 1999), where as CCL2 was revealed to enhance HIV-1 replication (Ansari et al., 2007). A natural mutation exists in the human population where individuals homozygous for the gene (CCR5-Δ32) are highly resistant to HIV-1 infection (Balotta et al., 1997). The mutation causes a 32 base pair deletion within the CCR5 gene and results in a lack of expression. However, infections are still possible in these individuals from X4 tropic and R5X4 dual-tropic viruses (Balotta et al., 1997; Gorry et al., 2002).

SDF-1/CXCL12 is the only known ligand for CXCR4. Six isoforms of CXCL12 have been identified. The splicing variant CXCL12γ was determined to be 6 times more potent in blocking HIV-1 infection compared to CXCL12α (Altenburg et al., 2007), which functions by ligand medicated receptor CXCR4 internalization (Altenburg et al., 2010).
1.1.6 Factors affecting HIV-1 expression

A major characteristic of lentivirus is its ability to remain dormant for many years before replication occurs. It has been found that there are nine times more latently infected T cells compared to the number of cells that are actively expressing HIV-1 in the blood of HIV-1 patients (Schnittman et al., 1989). Latency is often caused by a lack of cellular factors required for provirus activation.

HIV-1 replication is found to be inducible by both mitogens and antigens that lead to cell activation (Rosenberg & Fauci, 1991). When infected cells are exposed to activation stimuli such as phytohemagglutinin (PHA) from kidney beans and gram-negative bacteria cell wall lipopolysaccharide (LPS), a signal cascade occurs that results in the activation of NF-κB. The 5’ LTR of HIV-1 provirus contains two tandem binding sites for NF-κB just upstream to the transcription start site. NF-κB binding leads to provirus activation and gene transcription.

Co-infection with another virus can also affect the replication status of HIV-1. Additional elements present in HIV-1 5’ LTR, such as Sp1 and TATA box, can bind with cellular transcription factors after exposure to gene products from other viral infections. Enhanced expression was observed in CD4+ T cells co-infected with HIV-1 and herpes simplex virus, cytomegalovirus, human herpes virus and T cell leukemia virus. One mechanism for human herpes virus to increase HIV-1 infection is through the activation of Fc receptors (Laurence, 1990). It has been reported that Fc receptor is responsible for antibody-mediated HIV-1 uptake in vitro (Athanassakis et al., 2000; Homsy et al., 1989). Heterologous viral infection can also produce antigens leading to cell activation and increased de novo HIV-1 infection. However, the exact effect depends on the particular virus in question.

Other factors that can induce cell activation can also potentially enhance HIV-1 expression. Cytokines are small secreted molecules that are involved in signal transduction. Tumor necrosis factor-α (TNF-α) can increase HIV-1 expression in chronically infected T cell and monocyte cell lines (Poli et al., 1990b). TNF-α activates a transcription activator that binds to the NF-κB consensus sequence in HIV-1 5’LTR. Viral expression is initiated in a similar manner to T cell activation. Interlukin-6 and granulocyte-macrophage colony stimulating factor (GMCSF) are also found to up-regulate HIV-1 expression in macrophages (Poli et al., 1990a). The function of these cytokines is thought to be autocrine/paracrine in nature. Some evidence exists where HIV-1
infection induces the secretion of TNF-α in monocytes (Clouse et al., 1989). There are also cytokines that function in suppressing HIV-1 replication. Interferon-α reduces HIV-1 expression in both acutely and chronically infected cells by inhibiting viral budding. (Poli et al., 1989) Transforming growth factor-β can both suppress and induce HIV-1 expression depending on the presence of other stimuli (Poli et al., 1991).

1.1.7 HIV-1/AIDS pathogenesis

During the acute phase of HIV-1 infection, the first tissue infected by sexual intercourse in women is the cervical epithelium as suggested by studies with simian immunodeficiency virus (Zhang et al., 1999). Infection is then carried by dendritic cells to regional lymph nodes and then enters the blood stream. In men, the risk of HIV-1 sexual transmission can be reduced by circumcision, which decreases both the surface area for infection and the number of HIV target cells available (Dinh et al., 2011). Another site of initial HIV-1 infection is the rectal gastrointestinal tract. Acute infection is associated with depletion of CD4+ T cells in the mucosal-associated lymphoid tissues in the gastrointestinal tract (MALT) (Guadalupe et al., 2003), more commonly known as gut associated lymphoid tissue (GALT) (Li et al., 2005). Due to a lack of immune response initially, blood viremia can reach as high as 10 million copies per mL (Little et al., 1999). Destruction of mucosal tissue triggers immune response from the GALT that result in release of pro-inflammatory chemokines. Extensive recruitment of immune cells to the infection site determines the course for progressive immunodeficiency. The peak of blood viremia is usually reached after three to four weeks post infection (Little et al., 1999). After 6 months, most patients infected with HIV-1 enter an asymptomatic period of the disease, where the viral load is below 20,000 RNA copies per mL. This is the result of an HIV-1 specific CD8+ cytotoxic T cell response (McMichael et al., 2010). The production of neutralizing antibody can also be detected after 12 weeks of infection; however, this is usually ineffective at clearing the virus and instead forces the virus to undergo divergence mutations to create neutralization-resistant strains (Richman et al., 2003b; Wei et al., 2003b). After an average of 10 years of infection without treatment, the majority of patients progress to AIDS. AIDS is characterized by having a CD4+ T cell count below 200 cells per µL of blood and a lack of immune activity caused by destruction of the adaptive immune response.
The main clinical manifestation of HIV-1/AIDS disease progression is the loss of CD4+ T cells, which is the underlying cause for the opportunistic infections and cancer seen in patients. During early stages of infection, CD4+ T cell loss can be balanced by increased production (McCune et al., 2000). Eventually, the immune system will have lost its replenishing potential and CD4+ T cell population starts to decline. This has been termed by some, “immune exhaustion”. The exact mechanism of HIV-1 induced T cell cytopathology is not well understood. One possible mechanism is the disruption of host cell membrane integrity caused by extensive budding during HIV-1 viral formation. It has been found that gp120 presentation on cell membranes is a possible marker for apoptosis since it is expressed preferentially on apoptotic CD4+ T cells from lymph nodes of HIV-1 patients (Sunila et al., 1997). gp120 presentation can also occur on uninfected CD4+ T cells, which targets them for anti-gp120 antibody dependent cellular cytotoxicity involving CD8+ T cells and natural killer cells (Weinhold et al., 1989), the cells responsible for viral infection clearance (Zinkernagel, 1995). In addition, uninfected CD4+ T cells can simply be non-specifically targeted for apoptosis by a bystander effect (Zhang et al., 2001). The gradual loss of CD4+ T cells can also be attributed to the disruption of intrathymic maturation of precursor T cells due to HIV-1 infection thus impeding the regeneration of mature T cells (Dion et al., 2004).

Besides causing cell death, HIV-1 infection can also cause impairment of CD4+ T cell immune response. A decrease in antigen-specific responses has been observed in vitro in HIV-1 infected CD4+ T cells (Lane et al., 1985; Teteuwen et al., 1990). In the absence of infection, exposure to HIV-1 gp120, gp41 and p24 alone can result in a decreased immune response in vitro (Nong et et al., 1991; Ruegg & Strand, 1990). One mechanism being proposed is that HIV-1 proteins can interfere with the interaction between CD4 and MHC. Also binding of gp120 to cell surface receptors could also result in altered signaling pathways. Studies have also found HIV-1 can down-regulate the T cell receptor and IL-2 receptor alpha expression, which results in immune dysfunction (Johnson & Parkin, 1997; Willard-Gallo et al., 2001).

1.1.8 Drugs for treating HIV-1/AIDS

Since the discovery of HIV-1 as the causative agent for AIDS immense effort has been made to combat the disease. However, no cure has been found to date. Drugs have been developed
aiming at delaying disease progression by keeping blood viral load at a minimum. The first antiretroviral drug approved to treat HIV-1 was developed in 1987. Azidothymidine (AZT, zidovudine), a thymidine analogue, was shown to inhibit viral infectibility and reduce cytopathic effect of HIV-1 in vitro (Mitsuya et al., 1985). So far there are a total of 25 antiretroviral (ARV) drugs approved by the FDA to treat HIV-1 clinically (De Clercq, 2009). These ARVs function by targeting key steps within the HIV-1 life cycle and fall into one of four categories: fusion and entry inhibitors, reverse transcriptase inhibitors, integrase inhibitors and protease inhibitors.

Fusion and entry inhibitors stop HIV-1 infection at its initial contact site with the host cell membrane. Enfuvirtide is a 36 amino acid polypeptide used to block viral fusion to the host cell membrane (Matthews et al., 2004). It functions by participating in coiled-coil interaction with the heptad repeat region of gp41. Because enfuvirtide is a large peptide, it cannot be absorbed through the intestine by oral ingestion. It is given subcutaneously by injection twice daily and is primarily used in salvage therapy when other ARV treatments have failed. Co-receptor inhibitors are also used to block HIV-1 host-cell interaction. Maraviroc is the only approved CCR5 antagonist (De Clercq, 2009). However, it only prevents infection from R5 tropic viruses and may encourage the selection of X4 virus (Kromdijk et al., 2010). Thus it is best used in combination with a CXCR4 antagonist. A promising CXCR4 antagonist is the compound AMD3100 (Hendrix et al., 2000). However it is not orally available to use in conjunction with maraviroc. Therefore, an orally administrable CXCR4 antagonist is currently under development.

Reverse transcriptase inhibitors (RTIs) aim at preventing the synthesis of viral dsDNA from its genomic RNA. They are either nucleoside/nucleotide (NRTI/NtRTI) analogues that bind directly to the catalytic site of HIV-1 reverse transcriptase or non-nucleoside inhibitors (NNRTIs) that bind to an allosteric site on the enzyme (De Clercq, 2009). The NRTIs are 2’, 3’-dideoxynucleoside analogues. After they are taken into the body, three phosphorylation steps occur at the 5’end to convert them to the active form. They then function as competitive inhibitors for the normal deoxynucleoside triphosphates to stop incorporation or serve as a chain terminator. The difference between NtRTIs from NRTIs is that NtRTI already has one phosphate group attached. This phosphate group is non-cleavable by hydrolases, so the NtRTI cannot be removed again after incorporation. The prototype of NtRTI is the oral drug tenofovir, (R) 9-(2-phosphonomethoxypropyl) adenine, first characterized in 1993 (Balzarini et al., 1993). It remains
the most frequently used drug for treating HIV-1. The NNRTIs are all derivatives of the prototypes: HEPT and TIBO (De Clercq, 2009). Their allosteric binding site on RT is 15Å from the active site. Its close proximity to the active site interferes with substrate binding. However, the NNRTI binding site is prone to mutation, thus ongoing drug development is required.

The next event in the HIV-1 life cycle targeted for drug development is the integration of viral dsDNA into the host genome. The only drug approved as an integrase inhibitor (II) is raltegravir (Markowitz et al., 2007). It interferes with the strand transfer step of the integration process. Another potential integrase inhibitor is elvitegravir, which is currently in phase III clinical trials. So far, elvitegravir has been proven to be effective in reducing patient viral load with half the required dosage for raltegravir (Shimura & Kodama, 2009).

The last class of ARV drugs is the protease inhibitors (PI). Protease is essential in the maturation of newly formed HIV-1 virus. It functions by cleaving viral Gag and GagPol protein precursor and releasing individual mature proteins (Kohl et al., 1988). There are currently ten approved PIs for clinical use. Nine of them contain a hydroxyl ethylene scaffold. They mimic the peptide linkage that interacts with the viral protease catalytic site. Protease function is neutralized because these mimics cannot be cleaved themselves. Drug resistance has been observed for all PIs, which is attributed to two main reasons. First, great plasticity and polymorphism for the 99 amino acid HIV protease enzyme (Rhee et al., 2003) made it difficult to design a broadly reactive drug. Second, the lack of proof reading by the reverse transcriptase results in high mutation rates, which provides escape routes for the virus from treatment.

1.1.9 HAART

The current standard treatment for HIV-1/AIDS is Highly Active Antiretroviral Therapy (HAART). It is a combination of at least three antiretroviral drugs that usually consists of 2 NRTIs plus a PI/NNRTI/II. The aim of HAART is to reduce serum viral load to below detection levels and at the same time prevent rapid resistance development for individual drugs. Synergism is achieved with lower dosages to reduce the chance of harmful side effect. Viral replication is suppressed which allows the immune system to recover lost CD4+ T cells and, thus, slows down AIDS disease progression. HAART is a lifelong treatment, meaning it does not cure the disease,
and HIV-1 will appear again once the treatment is stopped. The combination of drugs used depends on the clinical history of the patient (e.g. treatment naïve), viral load and varies according to individual life circumstances. The regime typically involves taking different drugs at different times of the day and noncompliance can be an issue. However, fixed-dose combination drugs greatly reduce the number of pills a patient has to take each day. Failure of the treatment occurs when there is a consistent increase of viral load in the blood. This could arise from several reasons: failure to adhere to the treatment regime, medication side effects and development of drug resistance. When this occurs, the patient has to be switched to a salvage regime.

1.1.10 AIDS microbicides and vaccines

Research efforts have also been applied to prevention of HIV-1 transmission. Early topical microbicide development has been focused on non-specific viral inhibitor compounds such as surfactants that disrupt viral membrane (Shattock & Moore, 2003), polyanions that prevent attachment of the virus to host cell (Scordi-Bello et al., 2005) and agents that help to maintain an acidic pH in the vagina and prevent pathogen infection (D'Cruz & Uckun, 2004). However, the compounds that have reached clinical trials produced either inconclusive results or resulted in increased risk for infection compared to placebo (Skoler-Karpoff et al., 2008; Van Damme et al., 2002; Williams et al., 2007). This is the result of many unwanted side effects, such as inflammation of the mucosal lining that results in increased HIV-1 infection. The development of next generation microbicides has been focused on compounds that are more specific ARV drugs. Their specificity greatly enhances potency and efficacy, and reduces the chance for harmful side effects. Many of the drugs are similar to the ones already in use for the treatment of HIV-1/AIDS (Nuttall, 2010). The recent success of 1% vaginal gel formation of tenofovir in a phase IIb study shows promise to this field. A 54% reduction in infection rate was seen in KwaZulu-Natal, South Africa, women test subjects with high adherence (Abdool Karim et al., 2010).

The major obstacle that researchers in the field of HIV-1 vaccine development face is the high degree of diversity of the virus. Ongoing research aims to find a vaccine that can elicit an immune response in humans that will have a broad reactivity against different strains of HIV-1. Evidence of HIV-1 host cellular immune response comes from studies with patients with primary
infection (Goonetilleke et al., 2009; Koup et al., 1994). Cytotoxic CD8+ T cells have been detected that are specific for HIV-1 Gag, Pol and Env proteins. It is these cells that are responsible for selective killing of HIV-1 infected cells and the initial viral load reduction during acute phase of infection. Vaccine study using rhesus monkeys and simian immunodeficiency virus also suggested that factors stimulating T cell immune response aid in controlling viral load (Liu et al., 2009). However, the down side of cellular immune response is that it does not prevent infection.

The effect of humoral response in controlling HIV-1 infection and replication is more complex. Early antibody responses can be detected within one month of infection, however they are only neutralizing against homologous viral isolates (Richman et al., 2003a) and cross-reactive neutralizing antibodies are only developed with varying degrees during the chronic phase of infection (Brown et al., 2008). However, broad reactive neutralizing antibodies do exist in rare cases. They usually target the variable region and the CD4 binding site on gp120 also the transmembrane segment on gp41 (Sather et al., 2009). Studies have shown that the virus has evolved numerous ways to evade such response. The viral Env protein is heavily glycosylated, which blocks access of important epitopes from neutralizing antibodies (Kwong et al., 1998). The structure of the sugar can also be mutated rapidly to evade existing antibodies (Wei et al., 2003a). Finally, some of the epitope targets for broadly neutralizing antibodies exist only transiently during the viral fusion step thus makes an effective immune response a rare event (Frey et al., 2008). Recently three broadly neutralizing monoclonal antibodies have been identified in HIV patients that react to over 90% of circulating HIV isolates (Wu et al., 2010). Further analysis could help identify new effective strategies for vaccine development.

1.2 Glycosphingolipids (GSL)

1.2.1 Structure and classification

Glycosphingolipids are a subtype of lipid that is ubiquitously present in the outer leaflet of the plasma membrane of all eukaryotic cells. They comprise a hydrophobic ceramide backbone, which is a fatty acid chain linked to a sphingosine base, and a water soluble carbohydrate head group that is exposed to the extracellular space. Differences in the lipid moiety and the
composition and attachments of carbohydrate head groups result in great variability of the GSLs. Depending on their sugar moiety, GSLs can be subdivided into the neutral group or the acidic group. The neutral GSLs include monoglycosyl-, and oligoglycosylsphingoids and monoglycosyl- and oligoglycosylceramides. The acidic GSLs include the gangliosides and the sulfatides (The nomenclature of lipids. (recommendations 1976). IUPAC-IUB commission on biochemical nomenclature.1977). GSLs can also be sub-grouped according to their core sugar structure into the Ganglio- (GalNAcβ1-4Gal), Globo- (Galα1-4Gal), Lacto- (Galβ1-3GlcNAc) and neolacto-(Galβ1-4GlcNAc) series. A minor Gala series also exists where the first sugar added was a galactose using galactosyl ceramide instead of glucose.

1.2.2 Biosynthesis and degradation

The synthesis of GSLs begins when sphingosine is formed on the cytosolic surface of the ER, where the enzyme serine palmitoyltransferase (SPT) catalyzes the condensation of palmitoyl-CoA and serine (Braun & Snell, 1968). Ceramide synthase, encoded by one of six different genes present in humans, catalyzes an acylation reaction that adds a fatty acid chain with varying chain length and degree of saturation to form a dihydroceramide (Pewzner-Jung et al., 2006). The enzyme dihydroceramide desaturase then forms a 4, 5-trans double bond forming the ceramide (Geeraert et al., 1997). A sphingosine can be released by subsequently removing the fatty acid chain with a ceramidase. Currently, five human ceramidase genes have been cloned and their gene product functions under different pHs (Mao & Obeid, 2008). Ceramide can also be reformed by addition of fatty acyl-CoA which then travels to the Golgi though vesicular and non-vesicular transport (Funato & Riezman, 2001) for carbohydrate head group addition. Vesicular transport of ceramide to the Golgi from the ER involves the coat protein complex COPII, which is found to drive both cargo selection and vesicle formation (Watson & Stephens, 2005). Upon reaching the cytosolic face of cis-Golgi, glucosylceramide synthase/glycosyltransferase is responsible for the addition of glucose to the C1 position of ceramide (Coste et al., 1986). The lipid transport protein FAPP2 recognizes GlcCer in the Golgi and transports it back to the ER, where GlcCer is translocated from the inner leaflet to the outer leaflet of the ER membrane (D'Angelo et al., 2007). Alternatively, the ATP-binding cassette transporter MDR1 can flip glucosylceramide to the luminal surface of the Golgi (Lala et al., 2000), where all
complex GSLs will be synthesized (Lannert et al., 1998). On the luminal surface of the Golgi, lactosylceramide synthase/galactosyltransferase (GalT-2) adds a galactose in a β1-4 linkage to produce lactosyl ceramide (Chatterjee & Castiglione, 1987; Lannert et al., 1994), which is the precursor for all four series of GSLs.

GSLs are under constitutive degradation, which occurs in the acidic compartments of the endosomes and lysosomes (Sandhoff & Kolter, 2003). Degradation begins when domains of the plasma membrane are endocytosed within coated pits for recycling. Internalized vesicles rich in GSLs fuse with early endosomes, where GSLs destined for degradation becomes sorted into intra-endosomal vesicles (multivesicular bodies). Degradation occurs on intra-lysosomal vesicles where lysosomal glycosidases sequentially cleave off terminal carbohydrates from the non-reducing end. The lysosomal peripheral membrane is protected from degradation by highly glycosylated proteins called glycocalyx. GSLs with short carbohydrate head groups require help from sphingolipid activator proteins for degradation (Sandhoff & Kolter, 1996), since they need to be lifted out of the lipid layer to allow access of glycosidase enzymes. Deficiencies in either glycosidase enzymes or sphingolipid activator proteins can result in sphingolipid storage diseases in lysosomes. GSLs are eventually stripped of all sugar moieties and ceramide is produced. The ceramide is then deacylated by ceramidases to release sphingosine. Sphingosine is converted into sphingosine-1-phosphate (S1P) with sphingosine kinase inside the cytosol. Finally S1P is degraded by S1P lyase inside the ER to release hexadecenal and phosphoethanolamine. Alternatively, S1P can enter the salvage pathway, where S1P specific phosphatases (SPP) at the ER remove the phosphate group and the sphingosine is reused for new GSLs (Le Stunff et al., 2007).

1.2.3 Glycosphingolipids in health and disease

The composition of GSLs present in plasma membranes is cell type specific. The pattern of expression also changes with cell growth, differentiation, viral infection and oncogenesis (Hakomori, 1981). The tight regulation of GSL expression suggests they carry out important functions in the mammalian cell system. The carbohydrate group of GSL can be recognized by sugar binding lectins and other complementary carbohydrates (Hakomori, 2004). Their functions
on cell membranes generally belong to two aspects: adhesion/recognition and signal transduction.

The ganglio-series of GSLs are highly enriched in neural cells and are believed to be required for normal neurological functions. In a mouse knock out model, a lack of glycosylceramide synthase expression in the brain causes severe neural defects and results in death shortly after birth (Jennemann et al., 2005). In humans, mutations in the glucocerebrosidase (GBA1) gene results in Gaucher disease and development of Parkinson disease (Goker-Alpan et al., 2008). In addition Alzheimer disease has also been linked to reduced glycosylceramide synthase enzyme activity and decreased complex GSL composition in cell membranes (Marks et al., 2008). GSLs are also essential in the formation of water barrier in the skin (Jennemann et al., 2007). In fact, 4% of the total skin lipid mass is made up of glucosylceramide. Patients with severe Gaucher disease, in which GlcCer accumulates in lysosomes, often develop skin abnormalities.

In tumor cells, GSLs are found to be involved in growth, angiogenesis and metastasis, and their expression is often altered compared to normal cells. Ceramide is found to be a tumor-suppressor by activating cellular pathways that result in growth arrest and differentiation (Ogretmen & Hannun, 2004). Some chemotherapy aims to increase endogenous ceramide expression levels which then leads to ceramide induced apoptosis. Also lowered ceramide levels have been correlated with increased glucosylceramide synthase activity and the multi-drug resistant phenotype in cancer cell lines (Ogretmen & Hannun, 2001). Resistant cell lines often have three times higher glucosylceramide levels and enhanced MDR1 activity. Furthermore, in vivo experimentation has found tumor cell lines are able to shed the ganglioside GM1b into their environment to suppress anti-tumor immune response (McKallip et al., 1999). GSLs have also been found in higher concentrations in the apical membrane of the epithelial cells lining the stomach, intestine and respiratory tracts. These areas are often the first line of defense against foreign pathogen invasion. Unfortunately many pathogens have evolved mechanisms to exploit GSLs for their own pathogenesis. Membrane microdomains enriched in GSLs that have terminal Neu5Ac are the target for HIV-1, Ebola and H1N1 flu virus entry and budding (Manes et al., 2003). Bacteria such as Helicobacter pylori and Vibrio cholera also evolved ways to bind or modify terminal Neu5Ac GSLs for targeting host gastro-epithelial cells (Mahdavi et al., 2002; Smith et al., 2004). As a consequence, inhibitors for GSL biosynthesis or
metabolism offer new approaches for treating a number of infectious diseases (Kajimoto & Node, 2009).

1.2.4 GSLs and HIV-1

The classical HIV-1 infection paradigm involves the binding to CD4 on host cell with viral gp120, followed by binding to chemokine co-receptors and subsequent fusion. However, evidence from infections of CD4 negative cells has promoted the idea of an alternative pathway for HIV-1 infection. HIV-1 target cells including neural and colon epithelial cells express high levels of the GSL galactosylceramide (Harouse et al., 1991; Yahi et al., 1992), which has been found to bind with high affinity to gp120 (Bhat et al., 1992). This observation suggests GSLs can serve as an alternative receptor for HIV-1 in the absence of CD4. The binding site for GSLs on gp120 has been mapped to the variable region 3 (V3) (Cook et al., 1994). A V3 loop derived synthetic peptide SPC3 has been found to inhibit HIV-1 infection in both CD4 positive lymphocytes and macrophages (Yahi et al., 1994) and CD4 negative epithelial cells (Yahi et al., 1995). The receptors for SPC3 on cells have been found to be the GSLs: LacCer, GM3 and GD3 (Delezay et al., 1996). These GSLs share the common structural characteristic of a free hydroxyl group on position 4 of the terminal galactose. Studies measuring surface pressure in GSL monolayers revealed that gp120 from different HIV-1 isolates can penetrate GalCer and GM3 monolayers, but not monolayers made of ceramide, GlcCer or nonhydroxylated GalCer (Hammache et al., 1998). In addition, recent work from our lab has found that a soluble mimic of globotriaosylceramide, adamantyl Gb3, is a high affinity receptor for gp120 (Mahfoud et al., 2002). Infection experiment using human peripheral blood mononuclear cells (PBMCs) also confirmed that adamantyl Gb3 is effective in blocking HIV-1 productive infection (Harrison et al., 2010; Lund et al., 2006), suggesting the involvements of Gb3 during infection.

1.2.5 Lipid rafts and HIV-1

Lipid rafts are microdomains on the outer leaflet of plasma membranes that are enriched in sphingolipids and cholesterol. Their existence and function was first purposed by Brown and Rose (Brown & Rose, 1992). In model membranes, a lateral segregation propensity exists for
lipids to organize themselves according to their affinity for cholesterol (Lingwood & Simons, 2010). Sphingolipids, by having more saturated and rigid fatty acid chains, are attracted to the planar hydrophobic rings of cholesterol (Brown, 1998). Also, the connecting region between the head group and ceramide base of sphingolipids has greater ability to form hydrogen bonds to adjacent lipids compared to phospholipids. As a result, sphingolipids naturally cluster together to form the lipid ordered (L_o) phase with cholesterol inserted in between resulting in a state of local free energy minimum. These clusters are only nanometers in size and are very dynamic. They “float” within the larger pool of lipid disordered phase (L_d) which is enriched in phospholipids.

The existence of lipid rafts in membranes of living cells is, however, somewhat controversial. Because early purification methods for rafts from cells required treatment with non-ionic detergent like Triton X-100 and centrifuging through sucrose gradient, they were often criticized as experimental artifact. These isolated “rafts” are then described more accurately as detergent resistant domains (DRMs). The first strong evidence of lipid rafts in living cells has come from single particle tracking using colloidal gold conjugated antibody for raft glycolipid GM1 and associated protein Thy-1. They are found transiently restricted to areas of the membrane that are enriched in GSLs and resistant to Triton X-100 extraction (Sheets et al., 1997). Recent advances in microscopy techniques have given ample supportive evidences that lipid rafts are actual sub-compartments of the plasma membrane (Garner et al., 2008; Kim et al., 2008; Wesolowska et al., 2011). Common techniques to visualize rafts include fluorescence – cholera toxin B or anti-GM1 antibody labeling and microscopy.

Lipid rafts are functional units within the living cell due to enrichment of membrane proteins such as receptors and enzymes and act as dynamic platforms during signal transduction (Fielding & Fielding, 2000; Simons & Ikonen, 1997; Smart et al., 1999). The long and rigid saturated acyl chains of sphingolipids make rafts thicker than the rest of the membrane, and create a hydrophobic environment that is ideal for conformational changes that occur during signal transduction and enzyme activity. Protein lipid raft association is often regulated through post translational modification (Moffett et al., 2000). The common modifications include palmitoylation, myristoylation and addition of GPI anchors in the Golgi apparatus (Robbins et al., 1995; Rodgers et al., 1994). The trans Golgi network (TGN) is also the site for the formation of lipid raft precursors and DRMs can also be isolated from the Golgi (Gkantiragas et al., 2001). Thus the acylated proteins are likely to interact with sphingolipid and cholesterol enriched
domains in the TGN, then hitchhike the lipid raft vesicular transport pathway to reach the plasma membrane (Simons & Ikonen, 1997; Smart et al., 1999). Furthermore, the lipid raft structure is highly dynamic. Many smaller rafts can come together forming large patches in response to ligand binding to quickly concentrate enzymes for enhanced signal transduction. For example, T cell activation is precisely regulated by raft function, or autoimmune diseases can occur. During activation, the T cell receptor (TCR) binds with the MHC classes I/II molecule containing small peptides on APCs inside lipid rafts forming an immunological synapse (Drake & Braciale, 2001; Monks et al., 1998). Downstream signaling cascades then occur through tyrosine phosphorylation by recruitment of kinases (e.g. Lck) and exclusion of phosphatases (e.g. CD45), all of which could be regulated by lipid raft trafficking (Arni et al., 1996; Rodgers & Rose, 1996).

Because lipid rafts are important in cell function, many pathogens have evolved ways to utilize rafts to gain access to host cell machinery and exert pathogenicity. Examples include the influenza virus (Keller & Simons, 1998), measles virus (Vincent et al., 2000), Simian virus (Stang et al., 1997), *Vibrio cholera* (Wolf et al., 1998), verotoxin producing *E. coli* (Hanashima et al., 2008) and HIV-1 (Del Real et al., 2002; Liao et al., 2001). For HIV-1, lipid rafts are involved in multiple events through its life cycle. At the site of initial contact, the HIV-1 virus must first cross the mucosal lining of the gastrointestinal or genitourinary tracts through the process of transcytosis (Bomsel, 1997). This process relies on the binding of gp120-gp41 with the glycosphingolipid GalCer present in lipid rafts on the apical side of epithelial cells (Alfsen et al., 2001; Yahi et al., 1992). The raft trafficking pathway then transports the virus to the basolateral membrane. The main targets for HIV-1 productive infection are the CD4+ T cells and macrophages residing in the blood vessels underlining the mucosal barrier. The HIV-1 receptor CD4 and co-receptors CCR5 and CXCR4 are found to be co-localized with raft marker GM1 (Popik et al., 2002), and this association is required to support productive infection (Del Real et al., 2002; Kamiyama et al., 2009; Kozak et al., 2002). Also the HIV-1 gp160 directly interacts with lipid rafts, where the gp41 is found enriched in the raft fraction by extraction. The highly conserved LWYIK motif on the gp41 stalk located just prior to the transmembrane region recognizes cholesterol (Vincent et al., 2002). Monolayer membranes reconstituted from lipid rafts have shown to allow insertion of HIV-1 gp120, which was enhanced by the presence of CD4 (Fantini et al., 2000). This suggests lipid rafts as entry sites for HIV-1 virus. HIV-1
infection can also interfere with host cell signaling. The HIV-1 raft associated protein Nef is responsible for high viral load and rapid disease progression (Deacon et al., 1995; Kestler et al., 1991). Nef interferes with T cell activation by down-regulating surface expression of signaling molecules (e.g. CD4, CD28 and MHC-class I) and altering function of kinases (e.g. Lck, PAK2, PI3K), all of which may promote viral production (Greenway et al., 2003). The function of Nef is found to be dependent on its lipid raft association, which is controlled by myristoylation at its N-terminus and a number of signature motifs (Giese et al., 2006). Finally, evidence suggests that HIV-1 viral assembly and budding occur in lipid rafts (Bryant & Ratner, 1990; Nguyen & Hildreth, 2000; Rousso et al., 2000). The fact that the viral Env protein is palmitoylated and myristoylation of the viral precursor protein Pr55\textsuperscript{gag} promotes efficient assembly and budding further supports the critical role of lipid raft in HIV-1 replication.

1.2.6 Globotriaosylceramide (Gb\textsubscript{3}) and HIV-1

Gb\textsubscript{3} or P\textsuperscript{k} or the B cell differentiation marker CD77, is a glycosphingolipid that belongs to the P1P\textsuperscript{k} and GLOB blood group antigen systems of erythrocytes. The P1P\textsuperscript{k} antigen system contains three antigens, namely P1, P (Gb\textsubscript{4}) and P\textsuperscript{k} (Gb\textsubscript{3}). P and P\textsuperscript{k} are also named GLOB1 and GLOB2 respectively, which constitute the GLOB series of antigens together with GLOB3 (LUKE antigen or LKE) (Tippett et al., 1986). The glycosphingolipid nature of the P1P\textsuperscript{k} antigen system was discovered by Naiki and Marcus (Naiki & Marcus, 1974). Like all other glycosphingolipids, the P1P\textsuperscript{k}/GLOB antigens are synthesized systematically in the Golgi apparatus by the sequential addition of monosaccharides on a ceramide base. All of them arise from glucosylceramide and lactosylceramide. P\textsuperscript{k}/Gb\textsubscript{3} is a trisaccharide glycosphingolipid: Gal\,\alpha1-4\,Gal\,\beta1-4\,Glc\,\beta1-1Ceramide (Figure 1.4). It is synthesized by transfer of galactose from UDT-galactose to LacCer catalyzed by α-1-4 galactosyltransferase (Gb\textsubscript{3} synthase) encoded by the A4GALT gene (Steffensen et al., 2000). Most of the P\textsuperscript{k}/Gb\textsubscript{3} made is converted into P/Gb\textsubscript{4} by the enzyme β 1-3 N-acetylgalactosaminyltransferase (Gb\textsubscript{4} synthase) in the consumption of UDP-N-acetylgalactosamine (Okajima et al., 2000). Gb\textsubscript{4} is the most abundant glycosphingolipid present on red blood cell membranes by weight (Fletcher et al., 1979). The B3GALNT1 gene encodes for Gb\textsubscript{4} synthase and is expressed in many tissues, most predominately in the brain and heart (Okajima et al., 2000). However, tissues in the kidney, liver, spleen and stomach have very low
expression of Gb₄ synthase, thus cells in these locations retain high Gb₃ expression. Gb₄ is a synthetic precursor for LKE; and LKE has the putative structure of NeuAcα1-3Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1’Cer (Cooling et al., 1998). The P1 antigen is synthesized by a different pathway involving the paragloboside. The exact nature of the glycosyltransferase is unknown, but is suspected to be related to Gb₃ synthase due to identity in terminal sugar structures of P1 and Gb₃. Recent finding by Thuresson et al suggests the P1 expression could be a result of alternative splicing of a novel A4GALT exon (Thuresson et al., 2011). Figure 1.5 summarizes the Gb₃ synthesis pathway.

The human population expresses the P1Pₖ/GLOB antigens to variable degrees on erythrocytes giving different phenotypes. Firstly, the most common phenotypes are the P₁ (~80% in Caucasians and ~20% in Asians) and the P₂ (~20% in Caucasians and ~80% in Asians). Their difference being P₁ is P1 positive but P₂ is P1 negative (Landsteiner & Levine, 1928). The P/Gb₄ antigen is highly expressed in both phenotypes, but the Pₖ/Gb₃ antigen is only found in extremely small amounts, most likely due to its conversion to P/Gb₄. Secondly, the rare P₁ₖ and P₂ₖ phenotype individuals have high levels of Pₖ/Gb₃ and are P1 positive or negative respectively (Matson et al., 1959). Individuals with these phenotypes have mutations in the B3GALNT1 gene which result in a lack of Gb₄ synthase (Kijimoto-Ochiai et al., 1977). Thirdly, about 98.6% of Caucasians are LKE positive and have the P₁ or P₂ phenotype. The other 1.4% is LKE negative and has phenotypes similar to P₁ₖ or P₂ₖ; though they have slightly lower Pₖ/Gb₃ expression than regular Pₖ individuals (Cooling & Kelly, 2001; Tippett et al., 1986). Finally the rare phenotype p was observed where individuals do not have any of the P1Pₖ and GLOB antigens (SANGER, 1955). These individuals have mutations in the A4GALT gene and cannot synthesis Gb₃ and all subsequent glycosphingolipids.
Figure 1.4 – Globotriaosylceramide structure. $\text{Gb}_3 = P^k = \text{CD77} = \text{verotoxin receptor}$.

Figure 1.5 – Globotriaosylceramide ($\text{Gb}_3$) synthesis pathway.
Like other glycosphingolipids the P1P\(^k\) blood group antigens are found to be involved in human infectious diseases. \(Gb_3\) is the only receptor for the toxin produced by *Shigella dysenteriae* and Shiga like toxin/verotoxin produced by enterohemorrhagic *E. coli* (Ashkenazi & Cleary, 1989; Boyd & Lingwood, 1989; Lindberg et al., 1987). \(Gb_4\) has been found to be the receptor for the human Parvovirus B19 (Brown et al., 1994) and the target of auto-anti-P IgG produced during paroxysmal cold hemoglobinuria as a complication following bacterial or viral infections (Worlledge & Rousso, 1965). In the case of HIV-1, \(Gb_3\) has been proposed as an auxiliary receptor for infection in the literature. Studies conducted by Puri *et al* have found that CD4+ cells became resistant to HIV-1 gp120 mediated cell fusion with a gp120 expressing cell line TF228 when GSL biosynthesis was inhibited using PPMP (Puri et al., 1998). However, when erythrocyte \(Gb_3\) was reintroduced into these cell membranes, the fusion event was reconstituted with both X4 and R5 isolates of HIV-1 gp120 (Hug et al., 2000; Puri et al., 1999). The importance of HIV-1 gp120 V3 in binding with GSLs was then reported by Nehete *et al* (Nehete et al., 2002). Synthetic peptide mimicking residue 15 to 21 of the gp120 V3 loop was able to block HIV-1 entry from both X4 and R5 isolates. The binding sites for this peptide were found to be the GSLs: GM3, \(Gb_3\) and GalCer. \(Gb_3\) is also highly enriched in lipid rafts (Fantini et al., 2000). Therefore it was proposed that after initial binding of gp120 to CD4, further interaction with \(Gb_3\) by the now exposed V3 loop stabilizes the gp120 – CD4 complex and the lipid raft structure facilitates movement of the virion to a chemokine co-receptor, ultimately results in fusion of the virus to host cell (Fantini et al., 2000; Hammache et al., 1999; Nehete et al., 2002). Although these studies have revealed that \(Gb_3\) plays an important role in HIV-1 infection, they do not offer direct infection data, especially using CD4+ T cells, which support their hypothesis that \(Gb_3\) enhances HIV-1 productive infection. Indeed, the experiments were conducted using model fusion assays with transfected cells; they may not reflect real infection conditions. Thus it was necessary to have a second look at the role of \(Gb_3\) in HIV-1 infection.

To investigate the role of \(Gb_3\) during actual HIV-1 infection, our lab has conducted a series of studies using freshly isolated human PBMCs from individuals with different \(Gb_3\) expression levels. The results were surprisingly different than previous reports. The first hint that \(Gb_3\) may have a protective role against HIV-1 infection came from data using PBMCs from Fabry disease patients (Lund et al., 2005). Fabry disease results from lack of \(\alpha\)-galactosidase A, which is the enzyme responsible for breaking down \(Gb_3\) (Brady et al., 1967). Mutations in the \(\alpha\)-Gal A gene
result in misfolding and target the immature enzyme for ER associated degradation (ERAD). It is an X-chromosome linked genetic disease that leads to accumulation of Gb3 in lysosomes (Das & Naim, 2009). The study found that Fabry-PBMCs have great resistance to R5 HIV-1 productive infection, but no resistance was seen with X4 infections. However, cell surface Gb3 levels from Fabry-PBMCs were comparable to control cells but there was lower CCR5 plasma membrane expression in Fabry-PBMCs as well. Thus, it was concluded that the resistance seen with R5 HIV-1 infection, at least partially, was due to the scarcity of co-receptor CCR5 required for fusion, which may be a result of internal Gb3 accumulation. It was then found that an analog of Gb3, adamantyl-Gb3, where the fatty acid chain was replaced by a rigid cage like structure adamantane, can effectively block HIV-1 infection and fusion (Lund et al., 2006).

To further address the effect of Gb3 expression on HIV-1 infection, PBMCs from individuals with different P1Pk phenotypes were infected with R5 and X4 viruses (Lund et al., 2009). The results revealed that P1k PBMCs that have higher Gb3 expression had greatly enhanced HIV-1 resistance compared to control cells. On the other hand, p-PBMCs which are devoid of Gb3 were greatly more susceptible to infection than control cells. The difference in infection was solely due to Gb3 expression since HIV-1 receptor and co-receptor levels were similar between all groups. From these findings, our lab has hypothesized that Gb3 may be a natural resistance factor for HIV-1. To further support the hypothesis, the A4GALT gene was introduced into a Gb3 low expressing and HIV-1 infectable cell line HeLa (Lund et al., 2009). The resulting cells became resistant to subsequent HIV-1 productive infection. In contrast, when the cells were treated with small interfering RNA against A4GALT an increase in infection was observed. Another study using pharmacologic modulation of Gb3 levels also points to an inverse relationship between Gb3 expression and HIV-1 infection (Ramkumar et al., 2009). The drug 1-deoxygalactonojirimycin (DGJ) is an alkylated imino sugar, which is being developed as a chemical chaperon to treat Fabry disease, since it promotes correct folding of α-galactosidase A to evade ERAD (Asano et al., 2000; Hamanaka et al., 2008). It is also a competitive inhibitor for normal α-galactosidase A and prevents Gb3 catabolism. In this study, DGJ treatment was used to increase the Gb3 expression in the monocytic and X4 virus infectable cell line THP-1. This accumulation of Gb3 resulted in a significant increase in resistance to HIV-1 productive infection. However, no effect was seen when the same treatment was applied to a Gb3 negative sub-clone of THP-1 cells. In comparison, THP-1 cells were also treated with D-threo-1-phenyl-
2-palmitoyl amino-3-pyrrolidino-1-propanol (P4). P4 is a very potent inhibitor of glucosylceramide synthase and eliminates all subsequent GSLs (Jimbo et al., 2000). This treatment completely abolished Gb3 expression on THP-1 cells and greatly enhanced HIV-1 susceptibility. The effect of D8J and P4 on R5 virus infection was also tested using the glioblastoma cell line U87 transfected with CD4 and CCR5, and similar results were observed for R5 virus. Overall, an inverse linear relationship was found between Gb3 content and viral production. Because no effect was seen on cell surface expression of CD4 and HIV-1 coreceptors and cell viability, the difference in viral production was attributed to Gb3 levels alone.

As mentioned above, Gb3 is the only known receptor for the *E. coli* produced toxin, verotoxin (VT), which causes cell death by protein synthesis inhibition. Previous studies in our lab have shown a small amount of Gb3 was present in PHA/IL-2 activated normal human PBMCs by total GSL extraction (Lund et al., 2006). It was suspected that only a small fraction of the cells express the glycolipid. This would mean treatment of these PBMCs with VT should increase productive HIV-1 infection, due to elimination of Gb3. However, the results were opposite to what was expected. It seems a discrepancy exists, that we wish to investigate further in this thesis.

### 1.3 Verotoxins (VTs)

#### 1.3.1 Source and classification

*Escherichia coli* naturally colonize the gastrointestinal tract of human and ruminant animals. However, strains of *E. coli* that have acquired virulence factors in the form of plasmids can cause enteric diseases. They are divided into four groups: Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Enteropathogenic *E. coli* (EPEC), and the Enterohemorrhagic *E. coli* (EHEC) (Levine & Edelman, 1984). Verotoxins are produced by EHEC and over 380 different OH serotypes have been identified from patients infected with verotoxin producing *E. coli* (VTEC). The most common serotype in causing severe outbreaks and sporadic cases is the strain O157:H7 (Karmali et al., 2010). There are four major isotypes of VT: VT1, VT2, VT2c and VT2d. They can be produced either alone or in combination in an *E. coli* strain. VT1 is also called Shiga like toxin (SLT) since it is virtually identical in both structure and sequence to the...
Shiga toxin produced by *Shigella dysenteriae* type 1. Rabbit antiserum raised against Shiga toxin can be used to neutralize the toxin effect of patient isolated VT1 in HeLa and Vero cells (O’Brien et al., 1983). However, members of VT2 are serologically distinct from VT1 and cannot be neutralized by anti-Shiga toxin serum. Also VT2 is more frequently associated with EHEC induced human disease (Boerlin et al., 1999).

### 1.3.2 VT and human disease

Verotoxin producing *E. coli* (VTEC) was first recognized by the group of Konowalchuk *et al* in Canada in the late 1970s. They found that culture filtrates from *E. coli* strains isolated from infants with diarrhea had severe irreversible cytotoxic effect to Vero cells and speculated that VTEC is associated with diarrhea. In 1983, O’Brien and LaVeck purified a subunit toxin from the H30 strain of VTEC isolated by Konowalchuk, which they recognized to be very similar to Shiga toxin produced by *Shigella dysenteriae*. Hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) were later linked to VTEC strain O157:H7 in 1983 by the groups of Riley *et al* and Karmali *et al* (Karmali et al., 1983; Riley et al., 1983). Infection of VTEC usually occurs through the ingestion of contaminated meat products, water or vegetables. The onset of hemorrhagic colitis is characterized by severe abdominal cramps followed by watery diarrhea which is quickly replaced by bloody stools typical of lower gastrointestinal hemorrhage. Most patients recover from VTEC completely; however systemic complications can develop in young children, elderly and immune compromised patients resulting in HUS that is potentially fatal. HUS is defined by haemolytic anaemia, thrombocytopenia and acute renal failure (Palermo et al., 2009). The onset of HUS is found to be inversely correlated to an age related production of IgG antibodies against VT1 and VT2 (Karmali et al., 2003). There is no specific drug for curing HC and HUS, except treatments for managing the symptoms of the disease (Palermo et al., 2009). Today, EHEC remains a threat to human health, as reminded by the most recent outbreak in Germany where at least 784 patients were diagnosed with HUS and 23 deaths have resulted so far (Centers for Disease Control and Prevention, 2011).
1.3.3 VT structure

Verotoxin is a modular toxin with the $A_1B_5$ subunit structure. The wild type whole toxin is also termed holotoxin and has a molecular weight of 68,000 Da. It consists of a catalytic A subunit (MW: 32,000Da) and a receptor binding pentameric B subunit (MW: 7,700Da each), which are held together by non-covalent interactions. VT is also named Shiga like toxin (SLT) because VT1 is extremely similar to the Shiga toxin (Stx) produced by *Shigella dysenteriae*, with only one amino acid difference in their sequence (Takao et al., 1988). VT2 shares a 60% sequence similarity with VT1 (Fraser et al., 1994). All isotypes of verotoxin have the same overall 3D structure and function in the same manner. The ultimate effect of VT in target cells is protein synthesis inhibition by selectively removing the adenine 4324 residue from the 28S RNA in the 60S ribosomal subunit (Endo et al., 1988).

The N-glycosidase activity resides within the A subunit, which is cleaved into A1 and A2 fragments in the cell. The A1 portion has similar sequence and folding as the plant toxin ricin (Fraser et al., 1994). The catalytic site within A1 consists of the residues Tyr77, Tyr114, Glu167, Arg170 and Typ203 which create a binding pocket in the folded toxin structure (Fraser et al., 1994). The A2 fragment is covalently linked to A1 in the nonactivated toxin. The side chain of A2 residue Met260 extends into the catalytic site and blocks entry of substrates. A furin cleavage site (RXXR) sits between A1 and A2, which is cleaved during retrograde transport of VT through early endosomes. However, the cleaved A1 and A2 fragments are still held together by a disulfide bond between Cys279 and Cys286 and only come apart in the reducing environment of the ER (Fraser et al., 1994). The C-terminus of the A2 fragment forms an $\alpha$-helix that inserts into the central hole of the pentameric B subunit ring structure. Most of the interactions with the B subunits are within the A2 fragment, in the form of a four-stranded mix $\beta$-sheet, in addition to a $\beta$-hairpin structure from A1 fragment. The interaction between the A subunit and B pentamer is asymmetrical and only involves three of the five B subunits. Each B subunit is folded into one $\alpha$-helix and six $\beta$-strands. The $\alpha$-helices come together in the center of the ring and interact with the C-terminus A2 helix in an anti-parallel fashion, with the $\beta$-strands sitting on the outside of the ring (Fraser et al., 1994). The 3D crystal structure of Shiga toxin/verotoxin is shown in Figure 1.6.
**Figure 1.6** – Verotoxin structure. A: Side view of holotoxin (PDB: 1DM0). The location of disulfide bond is labeled: Cys242-Cys261. B: Schematic presentation of verotoxin. C: Pentameric B subunit (top view), with closest section of A subunit (Ala194 – Cys242, Pro258 – Met287). D: A subunit with locations of the five residues of catalytic site (Tyr77, Tyr114, Glu167, Arg170 and Typ203). Residue Met260 from A2 fragment is shown blocking entry to the active site. E: Position of Gb3 binding site 1 shown in pentameric B subunit. A total of 3 Gb3 binding sites have been proposed in each VT1B subunit (Ling et al., 1998) (Adapted from Fraser et al., 1994) and (Nyholm et al., 1996)).
1.3.4 VT host cell trafficking and effect

The cytotoxic effect of VT on target cells is dependent on its endocytosis and retrograde transport. The only known receptor for VT is the GSL Gb₃, which is recognized by the pentameric VT-B subunit (Lingwood, 1999). Upon binding, the VT-receptor complex is internalized through clathrin dependent and independent endocytosis (Khine et al., 2004; Romer et al., 2007; Sandvig et al., 1989). VT is then retrograde transported from endosomes to the ER via the Golgi network (Sandvig et al., 1992). The VT-A subunit is cleaved by furin into A1 and A2 fragments during transport from endosomes to the Golgi (Garred et al., 1995). These two fragments remain connected by a single disulfide bond, which becomes reduced upon reaching the ER. The now separated A1 fragment is activated and translocates into the cytosol to exert its ribosomal inactivating effect leading to protein synthesis inhibition. Figure 1.7 illustrates the retrograde transport pathway of VT inside target cells.

Three separate Gb₃ binding sites have been proposed on each B subunit, which results in a total of 15 potential binding sites (Ling et al., 1998). Two of these sites are absolutely required for VT uptake as identified by mutational study (Soltyk et al., 2002). Optimal binding of VT is achieved when all 3 sites are occupied by Gb₃. Several factors contribute to the optimal uptake of VT. First of all, it has been found that fatty acid chain length on Gb₃ affects VT binding and intracellular transport, where optimal binding requires a mixture of Gb₃ species (Pellizzari et al., 1992) and also depends on their special organization (Nyholm et al., 1996). The fatty acid chain length for Gb₃ varies from 16 to 24 carbons long and their distribution is cell type dependent (Raa et al., 2009). The most abundant species are in order: C24, C22 and C18/C16. C18 or C16 are short chain fatty acids, which have enhanced ability for VT retrograde transport ((Arab & Lingwood, 1998; Raa et al., 2009). Secondly, the orientation and local lipid environment (i.e. the presents of other lipid species) also affects VT binding (Arab & Lingwood, 1996). Recent studies in our lab have found that cholesterol can mask the availability of Gb₃ for binding of VT and HIV-1 gp120 (Mahfoud et al., 2010). In the study, VT was allowed to bind with model Gb₃/cholesterol (2:1) vesicles during equilibrium density gradient centrifugation. It was found that only a minor fraction of Gb₃ in low density vesicles was detectable by VT, where the majority of Gb₃ remained unseen. This masking effect was also observed in vesicles made from Triton extracts of Vero cells. However, fluorescence microscopy analysis had shown that cholesterol depletion from Vero cell membranes using methyl-β-cyclodextrin (MβCD) exposes
previously hidden pools of Gb$_3$ for VT binding. In addition, mixing of Gb$_3$ with the GSLs GalCer or GlcCer dampened the masking effect of cholesterol. It was later found that cholesterol induced GSL head group conformational changes to affect their availability for ligand binding (Lingwood et al., 2011). Thus, it was speculated that the difference in local lipid organization contributed to the difference observed in VTEC infection of adults and children (Khan et al., 2009). Thirdly, it was also speculated that VT could induce its own uptake. Concentration dependent uptake of VT was observed, where an increased rate of uptake resulted from addition of holotoxin (Torgersen et al., 2005). The presence of the A subunit was important in this process since the VT-B subunit alone had no effect. Thus it was proposed that the VT-A subunit may interact with some yet unknown targets on the cell surface to enhance VT internalization.

After the binding of VT to its receptor Gb$_3$, the VT-Gb$_3$ complex is endocytosed through multiple pathways. The most common route is through clathrin-coated pits (Sandvig et al., 1989), where the tyrosine kinase Syk was found to be involved (Lauvrak et al., 2006). VT induces clathrin heavy chain phosphorylation, which is abolished when the Syk activity was inhibited using siRNA, expression of a dominant negative mutant or treated with Syk inhibitor piceatannol. Special structures composed of lipid rafts called caveoli are responsible for a minor pathway used by VT to enter target cell (Khine et al., 2004). This pathway seems to be independent of Golgi transport since the Golgi retrograde transport inhibitor, brefeldin A, did not fully protect Vero cells from VT1 cytotoxicity. VT was also found to induce tubule formation originating in the caveoli, which could be involved in VT endocytosis (Hansen et al., 2009; Romer et al., 2007). The molecule dynamin was found to be important in the pinch off step in the formation of VT endocytic vesicles (Romer et al., 2007). Mutation of dynamin has been shown to reduce VT uptake by 60% in HeLa cells, which inhibits both clathrin dependent and independent pathways (Lauvrak et al., 2004).

The Golgi/ER transport of VT is required for the cytotoxic effect of the toxin. There are cell types that can bind and endocytose VT yet remain non-susceptible to VT cytotoxicity (Falguieres et al., 2001; Sandvig et al., 1992). A431 cells can be sensitized to VT by treatment with butyric acid, which cause an increase the fraction of VT transported to the Golgi and the ER (Lauvrak et al., 2004). The trafficking of VT to the Golgi network was found to be dependent on lipid rafts and GSL sorting processes, where both cholesterol extraction and GSL synthesis inhibition reduced VT Golgi transport (Falguieres et al., 2001; Raa et al., 2009; P. Tam et al., 2008). VT
bound to Gb, outside of lipid rafts was destined for degradation by lysosomes (Hoey et al., 2003). Also, the mitogen-activated kinase p38 was found to be activated by VT signaling, where inhibition of p38 function using siRNA protected HeLa cells from VT cytotoxicity (Walchli et al., 2008). p38 was found to be recruited to the endosomes where it formed a complex with β-arrestin (Skanland et al., 2009). Knockdown of β-arrestin increased Golgi transport of VT B subunit by 50%. Knockdown of p38 also increased VT Golgi transport and endosomal association of β-arrestin was lost, which suggested β-arrestin plays a regulatory role on p38 activity. Finally, VT was also found to be able to alter microtubule dynamics to facilitate vesicle trafficking (Hehnly et al., 2006). Small GTP binding proteins such as Cdc42 were found to be involved in this process (Hehnly et al., 2009).

The route to the ER from the Golgi network follows both COPI dependent and independent pathways. The COPI independent pathway is dependent on Rab6 (Girod et al., 1999) and Cdc42 (Luna et al., 2002). Also Yip1A and actin microfilaments are involved (Kano et al., 2009; Valderrama et al., 2001). After reaching the ER, the VT A1 fragment becomes dissociated from the rest of the toxin and is translocated into the cytosol. The exact mechanism is not understood, but the ER proteins HEDJ/ERdj3 and BiP may be important in the process (Yu & Haslam, 2005).
Figure 1.7 – Verotoxin endocytosis and retrograde transport. (Credit to Patty Tam with modifications)
1.3.5 VT as targeted treatment for cancer

The expression of Gb₃ in normal human tissues is greatly restricted and its exact function is unknown. Gb₃ is a marker during negative selection of tonsillar B cells during affinity maturation (Taga et al., 1997). However, most of the Gb₃ expression is seen in kidney epithelial and endothelial cells (Khan et al., 2009; Lingwood, 1994). Other areas of expression include: microvascular endothelial cells in intestinal lamina propria (Miyamoto et al., 2006; Schuller et al., 2007), platelets (Cooling et al., 1998) and subsets of germinal center B lymphocytes (Mangeney et al., 1991; Murray et al., 1985). Monocytes (van Setten et al., 1996), monocyte derived macrophages, dendritic cells (Falguieres et al., 2001), intestinal pericryptal myofibroblasts (Schuller et al., 2007), neurons (Obata et al., 2008) and endothelial cells in the central nervous system (Johansson et al., 2006). Important to emphasize is that surface expression of Gb₃ does not necessarily lead to VT cytotoxicity; retrograde transport is required for VT ribosomal inactivation (Jacewicz et al., 1994).

It has been found that many cancer cell types have elevated Gb₃ expression compared to normal counterparts. Examples include: Burkitt’s lymphoma (Fellous et al., 1984), B cell lymphoma (LaCasse et al., 1996), testicular cancer (Ohyama et al., 1990; Ohyama et al., 1993), colorectal carcinoma (Kovbasnjuk et al., 2005), ovarian cancer (Farkas-Himsley et al., 1995), breast cancer (LaCasse et al., 1999), gliomas (Arab et al., 1999), malignant meningiomas (Salhia et al., 2002) and acute non-lymphocytic leukemia (Cooling et al., 2003). In some instances, Gb₃ expression is not only increased in the cancer cell itself, but also in the tumor vasculature (Arab et al., 1997; Arab et al., 1999; Johansson et al., 2006; Johansson et al., 2009; Salhia et al., 2002). All of these findings lead to the proposal that VT could potentially be used as targeted treatment for cancers.

There are generally two approaches in using VT as a treatment for cancer. Either use the holotoxin to kill cancer cells directly, or use the VT-B subunit as a selective carrier for other cancer drugs. The only requirement for the first approach is that the target cells have to be able to support retrograde transport of VT. VT cytotoxicity is extremely potent, where it was estimated only one molecule released into the cytosol is able to induce cell death (Tam & Lingwood, 2007). Therefore, only small quantities of VT are required for treatment. Potential side effect could be the development of HUS. However, such cases are expected to be extremely rare and would only occur in the children and elderly. Using purified toxin is also safer since other
virulent factors are required for pathogenesis (e.g. LPS) (Palermo et al., 2009). To reduce potential negative effects of the treatment, controlled small doses could be delivered by direct injection into the tumor mass. Also VT1 should be used instead of VT2, since VT2 is often associated with severe cases of VTEC infections (Boerlin et al., 1999). In a mouse human malignant meningiomas xenograft study, complete tumor regression has been achieved 7 to 10 days after a single injection of VT1 (Salhia et al., 2002). Apoptosis has been observed in both the tumor cells and its vasculature. In another similar study, the animals were tumor free 60 days after the treatment and no side effects have been reported (Arab et al., 1999). In some studies, it has been reported that VT treatment is in fact more effective in multi-drug resistant tumor types (Arab et al., 1997; Arab & Lingwood, 1998; Arab et al., 1999; Farkas-Himsley et al., 1995). Therefore, VT holotoxin could be used in combination with chemotherapy to treat cancers of adults. The second approach uses VT-B subunit conjugated with cancer drug for targeted delivery. This eliminates the need for retrograde transport. However, potential threat is posed to normal cells that also express Gb3 as those mentioned above. To solve this problem, photo-activatable substances could be used (Amessou et al., 2008; Tarrago-Trani et al., 2006). It is still a long way before VT can be safely used clinically in treating cancer patients and a lot of research and development are needed.

1.3.6 Anti-viral effects of VT

Besides its antineoplastic property, VT has also been studied for its anti-viral activity. VT belongs to a group of ribosomal inhibitory proteins (RIP) that can be either plant or bacteria derived. They all share a similar mechanism of action, which depurinates a specific adenine in the 28S ribosomal RNA via an N-glycosidase activity. Other members of the RIP group, such as ricin, Poke-weed antiviral protein, *Pseudomonas* exotoxin-A and trichosanthin, are also being studied for their anti-HIV-1 properties with some *in vitro* success (Chaudhary et al., 1988; McGrath et al., 1989; Olson et al., 1991; Till et al., 1989). Most of the studies involve the toxins conjugated with monoclonal antibody against HIV-1 Env protein or receptor CD4. The aim is to selectively inhibit viral transcription without ill effects to normal host cell viability. Normally, the toxins are eliminated by neutralizing antibodies. Therefore, it is beneficial to identify different anti-HIV-1 RIPS for prolonged *in vivo* treatment.
A series of studies performed by the group of Hovde et al. at University of Idaho have found that VT provides anti-viral protection for ruminant animals. VT producing E. coli naturally colonizes the gastrointestinal track of ruminant animals without causing any disease as seen in humans. VT1 was first found to inhibit bovine leukemia virus (BLV) infection induced spontaneous lymphocyte proliferation in vitro (Ferens & Hovde, 2000). However, pokeweed mitogen response and IL-2 induced proliferation were not affected by VT. The VT1A subunit alone produced similar effects as the holotoxin in reducing BLV p24 expression. Treatment with a catalytically inactive mutant VT1A had no effect, suggesting the importance of protein synthesis inhibition. VT2 was then shown to have similar effects as VT1 in reducing viral production in BLV infected bovine PBMCs (Basu et al., 2003). Infected cells were shown to be permeable to 40-70kDa fluorescent dextrans, suggesting direct entry of VT in to these cells for selective killing. In addition, sheep infected with BLV was also shown to benefit from VTEC, where a high fecal VTEC count correlated with better prognosis (Ferens et al., 2006; Ferens et al., 2008). Finally, VT1A has also been shown to selectively induce apoptosis in bovine immunodeficiency virus (BIV) infected fetal bovine lung cells (Ferens & Hovde, 2007). Electron microscopy had revealed that VT1A interfered with all BIV-induced cell morphology changes, which could be the basis for viral replication inhibition.

Limited knowledge exists on the effect of VT on human immunodeficiency virus infection. The first hint for an anti-HIV-1 effect of VT came from the study performed by Farkas-Himsley et al. (Farkas-Himsley et al., 1991). A partially purified bacteriocin (PPB) was shown to selectively kill HIV-1 infected cell lines but had no effect on uninfected cells at the same concentration. At 10µg/mL PPB, HIV-1 p24gag production was reduced by 38% in infected cord-blood lymphocytes. The active ingredient in PPB was later identified to be verotoxin (Farkas-Himsley et al., 1995). The novelty of VT was that the toxin did not need to be conjugated to antibodies to target HIV-1 infected cells. Recent studies in our lab have found PHA/IL-2 activated human PBMCs to express low levels of the VT receptor Gb3. However, the specific cell type expressing Gb3 was not determined. Nevertheless, it is interesting to examine the effect of VT on de novo HIV-1 infection of PHA/IL-2 activated human PBMCs. Preliminary experiments suggest VT1 treatment of PBMCs during activation inhibits subsequent HIV-1 productive infection. This finding is counter intuitive based on our previous work that found Gb3 provides resistance to HIV-1 infection (Lund et al., 2009; Ramkumar et al., 2009). Indeed, VT treatment should have
killed all Gb$_3$ expressing cells, resulting in increased infection. Therefore, further studies were warranted.

1.4 **Rationale**

Despite years of intensive research, there is still no cure or any effective vaccine for treating or preventing HIV-1 infection. Previous experiments surprisingly found that treatment with VT1 during activation of PBMC can significantly reduce their susceptibility to subsequent productive HIV-1 infection. However, only a very small subpopulation of PHA/IL-2 activated PBMCs may actually express the verotoxin receptor Gb$_3$. The goal of this thesis was to explore the protective property of VT against HIV-1 infection and to characterize the effect of VT on HIV infection of PBMCs.

1.5 **Hypothesis**

Verotoxin produced from enterohemorrhagic *E. coli* can serve as a novel inhibitor for HIV-1 infection and/or replication.

1.6 **Specific Objectives**

1) To test whether the protective effect of VT against HIV-1 is receptor dependent using a Gb$_3$ negative T-cell line: JKT-C

2) Verify VT protection of PHA/IL-2 activated PBMCs from infection with an X4 lab strain HIV-1$_{III B}$ virus and to determine which VT subunit is responsible for protection

3) To determine the effect of VT on normal activated PBMC function by using a protein synthesis assay and gene expression array analysis

4) To select for a VT resistant subpopulation of the Gb$_3$/CD4 + monocytic THP-1 cell line and to determine how this selection would affect Gb$_3$ expression and HIV-1 susceptibility
2 Chapter 2: Material and Methods

2.1 Cell lines

The human T lymphocytes used: acute T cell leukemia Jurkat-FHCRC cells (JKT-C) (Schneider et al., 1977) were obtained from Dr. D.Branch (University of Toronto, Toronto, ON). The human acute monocytic leukemia cells (THP-1) (Tsuchiya et al., 1980) were obtained from NIH AIDS Research and Reference Reagent Program (Rockville, MD). Both cell lines were cultured in complete RPMI1640 media (Wisent Inc, St-Bruno, QC) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Oakville, ON), 100IU penicillin and 100µg/mL streptomycin (Wisent Inc) at 37°C in 5% CO₂.

2.2 Isolation and activation of peripheral blood mononuclear cells (PBMCs)

PBMCs were prepared as previously described (Lund et al., 2005). Briefly, approximately 32mL of fresh whole blood was obtained from healthy donors following informed consent in Yellow cap BD Vacutainer® blood collection tube containing acid citrate dextrose (ACD; BD, Franklin Lakes, NJ). The blood was mixed in a 1:1 ratio with complete RPMI1640 media and 32mL of the mixture was overlaid on top of 15 mL Ficoll-Paque PLUS (GE Healthcare AB, Stockholm, Sweden) in 50mL conical Falcon tube at room temperature (RT). Samples were centrifuged at 1,800 rpm (Accuspin™ 3R, Fisher Scientific) for 45 minutes. The middle buffy coat layer was collected and washed three times with Dulbecco’s PBS lacking MgCl₂ and CaCl₂ (Wisent Inc) and spinning at 1,200 rpm for 5 minutes. Viable PBMCs were then counted using Trypan Blue (Wisent Inc) (where approximate yield was 1 million cells per mL of whole blood) and re-suspended in complete RPMI1640 media to a concentration of approximately 1x10⁶ cells per mL. Phytohemagglutinin (PHA, from Phaseolus vulgaris Red kidney bean, Sigma-Aldrich) and interleukine-2 (IL-2, Sigma-Aldrich) were added to freshly isolated PBMCs to a final concentration of 50µg/mL and 10U/mL respectively for activation. The cells were then cultured at 37°C in 5% CO₂ and were ready for desired experiments on Day 4.

2.3 Verotoxins

Verotoxin-1 (VT1) was purified in Dr Lingwood’s lab from the recombinant Escherichia coli strain JB28 using a conventional column method as described (Nutiikka et al., 2003). Verotoxin-2
expressing *E. coli* was made in Dr James Samuel’s lab (Texas A&M University Health Science Center, College Station, Texas) and VT2 was purified in our lab as described (Tesh et al., 1994). VT1B subunit was purified from *E. coli* strain JB120 expressing cloned VT1B in our lab as described (Ramotar et al., 1990). VT1A subunit was a gift from Dr. Gariepy’s lab (Princess Margret Hospital, Toronto, ON). Alexa 488-VT1B was prepared in our lab using Alexafluor-488 tetrafluorophenyl (TFP) ester (Invitrogen) according to product manual.

2.4 Virus

The X4 HIV-1<sub>IIIB</sub> virus was provided by Dr. Branch’s lab (Canadian Blood Services). The stock virus was obtained from NIH AIDS Reference & Reagent Program (Rockville, MD) and grown in Jurkat-C cells. The stock was stored at -80°C and thawed at RT just prior to infection. Multiplicity of infection (m.o.i.) was calculated based on p24<sub>gag</sub> production levels in infected MT-4 cells (NIH AIDS Reference & Reagent Program) (Branch et al., 2002).

2.5 Verotoxin treatment and HIV-1 infection *in vitro*

Cells were treated with verotoxins before HIV-1<sub>IIIB</sub> infection. For Jurkat-C cells, VT1, VT1A or VT1B was added in the cell culture varying from 3 days to just 1 hour before infection. For PBMCs, VT1, VT1A or VT1B were added to the cells at the same time as PHA/IL-2 activation and kept in culture during the 3 day activation period before infection. A dose response experiment was also performed. Viable cells were counted using TrypanBlue and washed with complete RPMI1640 once before re-suspending in fresh media for infection.

HIV-1 infection was conducted in the Biosafety Level 3 Facility at the University of Toronto using the protocol described (Branch et al., 2002) with minor modifications. Approximately 5x10<sup>5</sup> cells were infected with X4 HIV-1<sub>IIIB</sub> virus (Jurkat-C: m.o.i. = 0.1; PBMCs: m.o.i. = 0.3; THP-1: m.o.i. = 0.4; n = 4) in a round bottom Falcon tube in a volume of 200 µL. Cells were incubated with the virus at 37°C in 5% CO<sub>2</sub> for 1 hour with occasional mixing. The cells were then washed 3 times with 2mL PBS lacking MgCl<sub>2</sub> and CaCl<sub>2</sub> and spun down at 1,000 rpm for 5 minutes. The cells were then re-suspended in 2mL complete RPMI1640 media (for PBMCs, the media was supplemented with 10U/mL IL-2) and cultured in 24 well plates at 37°C in 5% CO<sub>2</sub> for a week. Cell culture supernatants were collected and replaced with fresh media on day 3, 5 and 7 post-infection. Collected samples were stored at –80°C until ready for p24<sub>gag</sub> ELISA.
(ZeptoMetrix, Buffalo, NY) to measure viral production. p24\textsuperscript{gag} ELISA was performed according to ZeptoMetrix product manual.

### 2.6 VT cytotoxicity assay

Target cells were adjusted to a concentration of approximately $5 \times 10^5$ cell/mL and 200µL was dispensed into each well of a 96 well plate. 200 µL of VT1, VT2, VT1A or VT1B solution was added to each well in 10-fold serial dilutions. Cells were incubated for 4 days at $37^\circ$C in 5% CO\textsubscript{2}. On day 4, cell proliferation was quantified using alamarBlue\textsuperscript{®} assay (Biosource, Camarillo, CA). AlamarBlue is a REDOX dye indicator that becomes fluorescent at 590nm when reduced by cell metabolic activity and can be used to quantitatively measure the proliferation of cell cultures (Ahmed et al., 1994). The cells were carefully mixed and 90µL of cell sample was mixed with 10µL of alamarBlue\textsuperscript{®} in a fresh 96 well Falcon clear polystyrene tissue culture plate and incubated at $37^\circ$C in 5% CO\textsubscript{2} until the control sample (no VT) turned bright pink (approximately 3 to 4 hours). Media only was used as blank and remained blue. The fluorescence was measured using 540nm excitation wavelength and read at 590nm emission wavelength using Spectra MAX plate reader, Gemini EM (Molecular Devices). Proliferation was calculated as a percentage in comparison to no VT control (100%).

### 2.7 Gb\textsubscript{3}-low THP-1 subpopulation selection using VT1

A Gb\textsubscript{3}-low THP-1 subpopulation was selected using VT1 to investigate the relationship between Gb\textsubscript{3} expression and HIV-1 infection. Wild type Gb\textsubscript{3} positive THP-1 cells (WT THP-1) were treated with increasing concentrations of VT1 in 10x serial increments ranging from 1fg/mL to 10pg/mL. The cells were cultured in 24 well plates and kept at $37^\circ$C in 5% CO\textsubscript{2}. Cell culture was monitored for 6 days. The percentage of cell survival was determined on day 4 using alamarBlue\textsuperscript{®} assay. On day 6 the cell cultures that had a 10% cell survival rate were collected and washed with PBS. These cells were then re-suspended in complete RPMI 1640 media and allowed to expand at $37^\circ$C in 5% CO\textsubscript{2}. After approximately 4 weeks the cells were found to be actively proliferating by visual inspection. They were then challenged by another addition of 1pg/mL of VT1 to ensure VT resistance. The resulting cells were named selected THP-1 (selTHP-1) cells. A double selected THP-1 cell line was also made by incubating selTHP-1 cells with increasing concentrations of VT1 ranging from 10pg/mL to 1µg/mL in 10x serial increments. After 4 days, cell cultures treated with 1µg/mL of VT1 were found to have 10% cell
survival rate by alamarBlue assay. These cells were than collected and expanded as before. The resulting cells were named double selected THP-1 cells (d. selTHP-1).

2.8 Protein synthesis inhibition assay (PSI)

A $^3$H-Leu incorporation assay was performed to detect the effect of VT treatment on nascent protein synthesis. Approximately 6x10$^5$ cells were incubated in Leu-free DMEM media without serum (Specialty media, Phillipsburg, NJ) for 3 hours. Jurkat-C and THP-1 cells were treated with 1µg/mL VT1, VT1A or no VT for 3 hours. PBMCs were treated with 1µg/mL VT1A for 4 days during activation or for 3 hours on day 4 of activation. 5µCi of $^3$H-Leu (Amersham Biosciences, Buckinghamshire, UK) were added per sample for incorporation at 37°C for 30 minutes. Cells were then washed 3 times with PBS to remove unincorporated $^3$H-Leu. 10% trichloroacetic acid was used to precipitate protein. The protein was pelleted and then suspended in scintillation fluid. Radioactivity was detected using a beta-counter (Beckman LS6500, Bioanalytical Systems Group, Mississauga, ON) to quantify the amount of protein synthesis. Alternatively cell pellets were lysed and resolved by 12% reducing SDS-PAGE gel, and prepared for autoradiography as described (Mody et al., 1996).

2.9 VT antibody neutralization assay

VT1 and VT1A were pretreated with anti-VT1B (6869) antiserum to test whether their effect on PBMCs was B-subunit dependent, and therefore receptor Gb$_3$-dependent. Anti-VT1B antiserum was prepared in 10x serial dilutions ranging from 1:200 to 1:200,000 in complete RPMI 1640 media in 96 well plates. Equal volumes of VT1 or VT1A were then added to the antibody dilutions to give a final toxin concentration of 2µg/mL. The plate was then incubated for 30 minutes at 37°C. Treated media was then added to approximately 1x10$^5$ freshly isolated and PHA/IL-2 activated PBMCs to make a final toxin concentration of 1µg/mL in 48 well plates. The cells were then cultured at 37°C in 5% CO$_2$ for 3 days. On day 4 cell proliferation was measured by alamarBlue® assay.

2.10 GSL extraction and thin layer chromatography overlay detection of Gb$_3$

Cells were collected and washed twice with cold PBS lacking MgCl$_2$ and CaCl$_2$. The pellets were then extracted with 7.5mL of 2:1 chloroform:methanol (v:v) overnight with vigorous
shaking. The samples were then adjusted with PBS to produce a Folch partition (2:1:0.6 chloroform: methanol: water; C:M:W) (Dawson et al., 1996). Complete separation was achieved by centrifuging at 5,000rpm (Universal 16R, Hettich Zentrifugen) for 5 minutes. The lower organic phase was filtered through glass wool into a fresh glass tube, and then the solvent was evaporated under a gentle stream of nitrogen. Saponification was carried out overnight with 1M NaOH in methanol to degrade phospholipids. The pH was then neutralized with equal volume of 1N aqueous HCl and solvent volumes adjusted to a Folch partition. Upper phase was removed and the lower phase was washed twice with theoretical upper phase (C:M:W; 1:47:48) to remove salt. The lower phase was collected and dried down again.

Extracted lipids were re-suspended in 2:1 C:M to 10^6 cell/10µL and equivalent amounts were loaded onto plastic-backed silica TLC plates (Machery-Nagel, Bethlehem, PA). TLC plates were pre-run in 98:2 C:M to clear non-polar lipids, then separated in 65:25:4 C:M:W. GSL bands were visualized by orcinol spray (0.5% v/v in 3M H_2SO_4) which detects carbohydrates; and Gb_3 was specifically detected by VT overlay. For VT overlay, the TLC plate was blocked with 1% cold water fish gelatin in TBS for 1 hour at RT, then probed with VT1B (0.1µg/mL) in TBS overnight at 4°C with gentle shaking. After washing with TBS, the plate was then incubated with rabbit anti-VT1B antiserum diluted 1:2,000 in TBS for 1 hour at RT. After another washing step, HRP-conjugated goat anti-rabbit antibody was then added at a 1:2,000 dilution in TBS for 1 hour at RT. After washing, the plate was developed using 3mg/mL of 4-chloro-1-napthol (4-CN) in methanol freshly mixed with 5 volumes of TBS and 1:2,000 dilution of 30% v/v H_2O_2. Development was stopped by washing in water and air drying. The plates were scanned and band density was measured using ImageJ (NIH, version 1.43).

2.11 Flow cytometry analysis

Flow cytometry was used to detect cell surface receptors on THP-1 and PBMCs. Approximately 5x10^5 cells were incubated in 100µL of 10% mouse serum (Sigma-Aldrich) in FACS buffer (PBS, 2% v/v FBS, 0.1% w/v sodium Azide, 5mM EDTA) at 4°C for 30 minutes to block Fc receptors and non-specific binding. After spinning at 2,000rpm (Micromax, IEC) the cell pellets were then re-suspended in 100µL of FACS buffer containing 1µg of mouse anti-human CD3-FITC IgG2ak, CD14-APC IgG2ak or CD19-PE IgG1κ antibody and incubated at 4°C for 30 minutes in the dark. FITC mouse IgG2ak, PE mouse IgG1κ and APC mouse IgG2ak were
used as negative controls for gating. All antibodies above were purchased from BD Pharmingen, San Diego, CA. Cells were then pelleted and washed once with fresh FACS buffer and then diluted in 500µL of FACS buffer for data collection and analysis using FACSCalibur Analyzer or Becton Dickinson LSRII (Flow cytometry facility, Toronto MarS Building) equipped with Cell Quest® or FACSDeva® 3.0 software. For CXCR4 staining a panel of three antibodies was used. Cells were blocked with 10% normal goat serum (Vector Laboratories Inc, Burlingame, CA) then incubated with 0.5µg of mouse anti-human CXCR4 primary antibodies: 12G5 (eBioscience, San Diego, CA), MAB 173 (clone44717, NIH AIDS), or MAB171 (clone 44708, R&D systems, Burlington, ON) in 100µL FACS buffer. After washing once with FACS buffer, the cells were incubated with 0.3µg of secondary goat anti-mouse-Alexa-488 antibody (Invitrogen) in 100µL FACS buffer for 30 minutes at 4°C in the dark. A sample stained with secondary antibody only was used as negative control. For Gb3 labeling, cells were directly labeled with 5µg/mL VT1B-Alexa 488 for 30 minute at 4°C in the dark. Specificity of Alexa-488-VT1B was proven by lack of binding to the Gb3 negative cell line Jurkat-C.

2.12 Fluorescence microscopy

For epifluorescence microscopy, THP-1 cells were stained for Gb3 and CXCR4 in the same method as for flow cytometry. Cells were then fixed in 4% w/v paraformaldehyde in PBS for 10 minutes on ice then washed twice with PBS. Nuclei were labeled by incubating in 300nM DAPI in PBS for 5 minutes at RT. After washing twice with PBS, the cell pellets were suspended in a small volume of ProLong Gold antifade reagent (Invitrogen) and mounted onto glass slide. Epifluorescence images were acquired with Zeiss Axiovert 200 inverted fluorescence microscope with Volocity software (Imaging Facility, Hospital for Sick Children, Toronto, ON). All images were taken with constant exposure time and exported as TIFF file from Volocity. Color was added using PhotoshopCS5.

2.13 Total RNA extraction

Freshly isolated PBMCs were activated with PHA/IL-2 and grown either with 1µg/mL VT1A or no VT as control for 4 days. On day 4, the cells were collected for total RNA extraction using TRIzol® reagent (Invitrogen). Briefly, approximately 5 ~ 10x10⁶ cells were lysed by repetitively pipetting with 1mL of TRIzol followed by 5 minutes incubation at RT. Then 0.2 mL of chloroform was added and the sample was shaken vigorously by hand for 15 seconds. After 3
minutes of incubation at RT the sample was centrifuged at 11,000g for 10 minutes at 2°C. The white interphase between the upper aqueous and lower phenol chloroform phase was collected and incubated with 0.5mL isopropyl alcohol for 10 minutes at RT. The precipitated RNA was spun down as before, washed once with 75% ethanol and centrifuged at 7,500g for 5 minutes at 4°C. RNA was re-dissolved in 0.01% diethylpyrocarbonate water by incubating at 55°C for 20 minutes. Concentration was determined by measuring $A_{260}$ and quality of RNA was assessed by having an $A_{260}/A_{280}$ ratio of ~2 (Cathala et al., 1983). Samples were stored at –80°C until further analysis.

2.14 Human gene expression array analysis

Purified total RNA samples were submitted to The Centre for Applied Genomics (TCAG, The Hospital for Sick Children Research Institute) for human gene expression array analysis. Total RNA was treated and amplified using the Illumina® TotalPrep™-96 RNA Amplification Kit (Ambion, Auston, TX). Briefly, total RNA was first reverse transcribed into single stranded cDNA. Then the second DNA strand was synthesized and the double stranded cDNA was purified using magnetic beads. The dsDNA was transcribed into Biotin-labeled cRNA in vitro. Finally, the resulting cRNA was purified by capturing with RNA binding beads.

Human WG-6 Expression BeadChip from Illumina® (San Diego, CA) was used as the array platform. The results were analyzed at the Statistical Analysis Core Facility (The Hospital for Sick Children Research Institute, Toronto, ON) with the assistance of Dr. Pingzho Hu.

2.15 Statistics

For microarray analysis, background correction was done with BeadStudio software (Illumina). The quantile normalization method implemented in lumi R package was used to normalize the data quantiles (Du et al., 2008). Differentially expressed genes were identified using LIMMA (linear models for microarray data) (Smyth, 2004). Briefly, a linear model was fitted for each gene in the data, and then an empirical Bayes (EB) method was used to moderate the standard errors for estimating the moderated t-statistics for each gene, which reduced the standard errors towards a common value. The corresponding p-values for the t-statistics were adjusted using the multiple testing procedure. Differentially expressed genes were identified by having a fold change >2 and adjusted p-value < 0.03.
HIV-1 infection p24\(^{gg}\) ELISA data and VT cytotoxicity assays were represented as the mean of several separate experiments, where n was indicated in the figure legends. Error bars represent standard error mean (±/ SEM). A two-tailed student’s t-test was performed where appropriate and p-values less than 0.05 were considered significant (*) and p-values less than 0.03 were considered highly significant (**).

### 2.16 Western blot

Western blot was used to examine the global cell activation state of PBMCs by detection of phospho-tyrosine expression pattern. Freshly isolated PBMCs were activated with either PHA/IL-2 (as previously described) or phorbol-12-myristate-13-acetate (PMA; AG Scientific, San Diego, CA). PMA, a phorbol ester derived from plants is an activator of protein kinase C and promotes cell growth in vitro (Blumberg, 1988). On day 4 post-activation, cells were collected and lysed for 10 minutes on ice. The post nuclear fraction was obtained by spinning the lysate at 17,000g for 15 minutes. Protein concentration was determined using BCA protein assay (Pierce, Rockford, IL) with BSA as standard. The lysate was then loaded and separated on a 10% reducing SDS-PAGE gel with each lane containing 20\(\mu\)g of total protein. The proteins were transferred onto nitrocellulose membrane for 1 hour at 100V (Whatman, Dassel, Germany). The membrane was blocked overnight at 4°C with 5% bovine serum albumin (Sigma-Aldrich) in PBS. Phospho-tyrosine was then detected using 4G10 antibody (gift from Dr. Wrana’s lab, Mount Sinai Hospital, Toronto) at a dilution of 1:10,000 in PBS overnight. The membrane was washed with three 5 minute washes in PBS-0.1% Tween20, followed by 1 hour incubation at RT with HRP conjugated goat-anti-mouse secondary antibody. After washing four times with PBS-0.1% v/v Tween20, the membrane was developed with HyGLO\(^{TM}\) chemiluminescent HRP antibody detection reagent (Denville Scientific, Saint-Laurent, QC) and exposed to autoradiography film. The membrane was then stripped by washing three times (20 minutes each) in stripping buffer (0.1M glycine, 0.1% SDS w/v, 1% Tween20 v/v, pH 2.2) and re-probed with anti beta-tubulin (clone E7, Hybridoma Bank, University of Iowa) antibody at a dilution of 1:8,000.

Total CXCR4 expression in THP-1 cells was also detected using Western blot. A similar method was used as described above; except mouse anti-hCXCR4 clone 12G5 (eBioscience) diluted 1:10,000 in 5% BSA in PBS was used as primary antibody.
2.17 Histone deacetylase inhibitor SAHA treatment

Suberoylanilide hydroxamic acid (SAHA; Vorinostat; Selleck Chemicals, Houston, TX) was used to study whether histone packaging reduces HIV-1 susceptibility of activated PBMCs. Gene expression microarray result indicated that VT1A treatment produced elevated histone protein gene expression levels, which could have induced tighter chromatin packaging. SAHA is a histone deacetylase inhibitor, which promotes histone acetylation. This process results in histone dissociation from chromatin DNA and potentially counteracts the effect of increased histone expression. 2.5μM of SAHA (Reuse et al., 2009) was added to PBMCs either at the time of activation or on Day 3 post activation. This treatment was combined with or without 0.5μg/mL of VT1A as indicated in figure legend. On day 4, SAHA and VT1A were washed away using RPMI1640 media. Cells were then infected with HIV-1ⅢB (m.o.i. = 0.3). 2.5μM of SAHA was re-added to the SAHA treated cell cultures on Day 0 and Day 3 post infection.
Chapter 3: Anti-HIV-1 effect of Verotoxin in human T cells

3.1 Summary

Pilot experiments from our lab showed VT1 treatment of activated human PBMCs significantly induced resistance to subsequent HIV-1$_{\text{IIIb}}$ infection. In this study a Gb$_3$ negative T cell line Jurkat-C was used to investigate the dependency of the anti-HIV-1 effect of VT on its receptor Gb$_3$. The cell line was confirmed to be Gb$_3$ negative using total neutral glycosphingolipid extraction and VT overlay TLC. Treatments with VT1B and the alpha-Gal A inhibitor DGJ were unable to induce detectable accumulation of Gb$_3$. JKT-C was then confirmed to be resistant to VT cytotoxicity and protein synthesis inhibition effect. Surprisingly, VT treatment significantly reduced productive HIV-1$_{\text{IIIb}}$ infection in JKT-C, even with just 1 hour incubation prior to infection. When the cells were treated with individual subunits of VT, only the catalytic VT1A subunit was able to confer viral production reduction. The efficacy of VT1A was comparable to the VT1 holotoxin. These results imply the antiviral property of VT is independent of its receptor Gb$_3$ and protein synthesis inhibition in JKT-C. However, upon repetition it was found that viral production of infected JKT-C greatly depended on culture passage and the results were variable between experiments. Therefore it was necessary to further study the anti-HIV-1 properties of VT in primary PBMCs.

PHA/IL-2 activated PBMCs were confirmed to be comprised of over 97% CD3+ T cell blasts using flow cytometry. Only approximately 3% of PBMCs were found to be weakly Gb$_3$ expressing as shown by $^{14}$C-galactose metabolic labeling and flow cytometry. Infection experiments revealed that both VT1 holotoxin and VT1A subunit treatments significantly reduced subsequent HIV-1$_{\text{IIIb}}$ productive infection, similar to the results seen in JKT-C. However, a reduction in cell proliferation was observed in VT treated samples in comparison to no VT control samples. A dose response infection experiment with VT1A showed that viral production appeared to be dependent on cell proliferation rate.

Upon further investigation, PHA/IL-2 activated PBMCs were found to be susceptible to the cytotoxic effect of VT despite their extremely low Gb$_3$ expression level. A dose dependent cell proliferation reduction was observed, where the VT1A subunit was more potent compared to the holotoxin with LC$_{50}$ values of 10nM and 40nM respectively. This cell proliferation inhibition
The effect was further confirmed to be Gb\textsubscript{3} independent using a VT1B neutralization assay. It was also found that VT1A treatment did not affect the ratio of CD3+/CD4+ vs. CD3+/CD8+ T cell composition and no significant changes in global phospho-Tyr protein expression pattern of the activated PBMCs were observed. However, protein synthesis was reduced up to 45% after VT1A treatment which could be a consequence of proliferation reduction. Finally, gene expression array analysis revealed that VT1A treatment caused an up-regulation of histone cluster protein mRNAs. This could result in increased chromatin histone content and lead to inhibition of viral genome integration or viral gene expression. Overall, the results suggest the anti-HIV-1 property of VT1 in PBMCs is achieved through cell proliferation arrest and reduction in protein synthesis. However, the mode of action of VT1 is independent of its receptor Gb\textsubscript{3}. The exact route of entry is still unclear but is speculated to be random fluid phase macropinocytosis.

\section*{3.2 Background}

Verotoxin or Shiga like toxin is an AB\textsubscript{5} subunit toxin produced by enterohemorrhagic \textit{E. coli}. VT belongs to a group of ribosomal inactivating proteins that inhibits protein synthesis in target cells by specifically removing an adenine residue in the 28S rRNA with its N-glycosidase activity (Endo et al., 1988). Ruminant animals naturally harbor EHEC in their digestive system without any harmful effect (Beutin et al., 1993) and benefit from their anti-viral effect. The enzymatic A subunit of VT have been shown to inhibit expression and replication of two bovine retroviruses, bovine leukemia virus (BLV) and bovine immunodeficiency virus (BIV) (Ferens \& Hovde, 2000; Ferens et al., 2006; Ferens \& Hovde, 2007). A major characteristic of BLV infection in bovine PBMCs is the spontaneous lymphocyte proliferation \textit{in vitro}. VT1 treatment has been shown to inhibit this process without any cytotoxic effect or altered response to normal immune stimulants (Ferens \& Hovde, 2000). Also the catalytic activity of the A subunit is required for the inhibition effect since the catalytically inactive mutant VT1A, E167D, was ineffective. Furthermore, the BLV p24 core protein expression was reduced by VT1A treatment. However, this reduction was only seen in the cell associated fraction but not in the culture supernatant, which suggests VT1A specifically eliminates BLV infected cells by lysis (Basu et al., 2003). Studies with BIV also suggest VT specifically inhibits viral production by inducing apoptosis in infected cells (Ferens \& Hovde, 2007). However, no direct binding of VT to bovine PBMCs or BLV has been detected (Basu et al., 2003). Since BLV infected bovine B cells showed greatly increased uptake of macromolecules of up to 70kDa, the specificity of VT to
infected cells is thought to be a result of increased cell membrane permeability caused by viral infection (Basu et al., 2003).

Extensive studies have been done on the antineoplastic properties of VT since many human tumor cell types express VT receptor Gb3 (Gariepy, 2001; Lingwood, 1999). However, very limited results exist on the effect of VT against human HIV-1 infection. It has been found that VT specifically kills HIV-1 infected human cord blood lymphocytes and T cell lines (Farkas-Himsley et al., 1991). Increased DNA fragmentation and cell debris was observed in HIV-1 infected cells but not in uninfected cells. Peripheral blood mononuclear cells are often used to study human HIV-1 infection in vitro. Activation of PBMCs with the T cell mitogen phytohemagglutinin (PHA) and IL-2 results in over 95% proliferating T cell blasts (Branch & Mills, 1995). Pilot experiments in our lab have found that VT treated PHA/IL-2 activated human PBMCs have significantly reduced productive HIV-1 infection. It was later found that activated PBMCs only express a very low amount of Gb3 (Lund et al., 2009; Ramkumar et al., 2009). Thus it was unclear how VT treatment of PBMCs could induce such a dramatic effect against HIV-1 infection.

In this current study the human acute leukemia T cell line Jurkat-C was used to determine whether the anti-viral effect of VT is dependent on its only receptor Gb3. JKT-C is both CD4 and CXCR4 positive and susceptible to T cell tropic virus infection including HIV-1IIIB (Wendler et al., 1987). Since JKT-C does not express Gb3, VT is expected to have no effect on JKT-C cell functions, including proliferation and protein synthesis. HIV-1 susceptibility of JKT-C cells should not be affected if the anti-viral property of VT is Gb3 dependent. In addition, the anti-HIV-1 effect of VT was further elucidated in PHA/IL-2 activated PBMCs. The cell type composition and Gb3 expression levels of activated PBMCs were first characterized. The T-cell tropic HIV-1IIIB virus was used for infection to verify and extend previous results. Also the subunit requirement and receptor dependency of the anti-HIV-1 effect of VT were determined using individual isolates of VT1A and VT1B subunit. The effect of VT on normal PBMC cell functions such as cell proliferation, protein synthesis, general activation state, and gene expression were examined to determine its mode of action in inhibiting productive viral infection.
3.3 Results

3.3.1 Anti-HIV-1 effect of VT in Gb3 negative human Jurkat-C T cell line

3.3.1.1 Testing Jurkat-C cells for Gb3 expression

It was first necessary to verify that Jurkat-C cells were completely Gb3 negative. This was confirmed using total neutral glycosphingolipid extraction and VT overlay Gb3 detection. Cells were first treated with DGJ (1-deoxygalactonojirimycin) with or without VT1B for 5 days. The treatment was intended to prevent the breakdown of any Gb3 that might be made in the cell. DGJ is a competitive inhibitor of alpha-galactosidase A (alpha-Gal A) (Asano et al., 2000), the enzyme responsible for Gb3 degradation, which would lead to accumulation of Gb3. The result (Figure 3.1) showed that up to 200µM of DGJ and 1µg/mL of VT1B together was unable to induce any detectable accumulation of Gb3 in JKT-C cells.

![Gb3 detection](image)

**Figure 3.1** – Jurkat-C cells do not express Gb3. JKT-C cells were treated with 50µM, 100µM or 200µM of DGJ with or without 1µg/mL of VT1B for 5 days. Samples were subjected to neutral glycosphingolipid extraction and resolved on TLC plate. VT overlay was used to detect Gb3. A Gb3 naturally expressing cell line THP-1 was used as positive control. Each lane represents extraction from $3 \times 10^7$ cells.
3.3.1.2 Verotoxin sensitivity of Jurkat-C cells

Verotoxin belongs to a group of ribosomal inactivating proteins. Upon binding to its receptor Gb₃ in susceptible cell types, the toxin is internalized into the cell and transported retrogradely into the ER (Sandvig et al., 2004). Protein synthesis is stalled as a result of ribosomal inactivation by depurination which results in cell death. Since JKT-C cells are not Gb₃ expressing, VT treatment should not have any cytotoxic effects on JKT-C cells and would not be able to inhibit protein synthesis as seen in VT susceptible cell types. This was confirmed by cell proliferation assay using alamarBlue® after VT treatment and protein synthesis inhibition assay using ³H-Leu incorporation.

JKT cell proliferation after VT treatment was measured using alamarBlue® REDOX dye indicator system. Cell metabolic activity changes the dye from blue to pink and causes it to become fluorescent at 590nm. The intensity of fluorescence is proportional to viable cell density as shown in Figure 3.2 a, which was linear when cell density was above 16x10⁴ cell/mL. JKT-C cells were then treated with VT1 or VT1B in 10x serial dilutions for 4 days. The result showed that VT1 was non-cytotoxic to JKT-C cells in the range of 0.1ng/mL to 1µg/mL (Figure 3.2 b) since no detectable reduction in cell proliferation was seen comparing to no VT control samples. JKT cell viability after VT treatment was also tested by using TrypanBlue exclusion assay (Figure 3.2 c). There was no difference in cell viability between no-VT control and VT1, VT2 or VT1B treated samples. The potency of VT was confirmed using the Gb₃ positive cell line THP-1 (Figure 3.2 d). Only 0.1ng/mL of VT1 was enough to completely inhibit THP-1 cell proliferation; however the cells were unaffected by VT1B treatment.

The effect of VT on JKT-C protein synthesis was tested using ³H-Leu incorporation assay. The primary route of action of VT in causing cell cytotoxicity is through depurination of ribosome and stalling protein synthesis. Decreased protein synthesis is a rapid indication of whether VT has any adverse effect to JKT-C normal cell function and whether VT is transported to the interior of the cell. Jurkat-C cells were treated with or without VT (see figure legend) for 4 days and then incubated with Leu-free media for 3 hours. ³H-Leu was added for 30 min at 37°C then removed by washing with PBS. The amount of nascent protein synthesized during that period was measured by beta-counting and visualized by autoradiography after separation by SDS-PAGE (Figure 3.3). The results showed there were only a 7%, 9% and 5% reduction in ³H-Leu
incorporation after VT1, VT2 and VT1B treatment respectively. Statistical significance with p < 0.05 was only observed with VT1 treatment. Autoradiography revealed no noticeable changes in the general pattern of global nascent protein synthesis. Therefore VT appears to have minimal effect in interfering with JKT-C protein synthesis machinery.

3.3.1.3 VT can significantly reduce HIV-1\textsubscript{IIIb} productive infection in JKT-C

\textit{In vitro} HIV-1\textsubscript{IIIb} infection (m.o.i. = 0.1) experiments were performed to determine whether VT treatment can reduce viral production in Gb\textsubscript{3} negative JTK-C cells. JKT-C cells were treated for 3 days with VT1 or VT2, the same duration that was used to treat PBMCs in pilot experiments. VT concentration was chosen to be 1µg/mL based on the proliferation assay result to give maximum effect. The VT was removed prior to infection with HIV-1\textsubscript{IIIb} by washing once with RPMI media. Viral production was measured using p24\textsuperscript{Gag} ELISA. If the protective effect of VT against HIV-1 is receptor Gb\textsubscript{3} dependent, no reduction in viral production would be observed. The results showed that VT1 and VT2 treated cells had reduced viral production following HIV-1\textsubscript{IIIb} infection (Figure 3.4 a). There was a 39% reduction of p24 production after VT1 treatment and a 90% reduction after VT2 treatment with p < 0.05. This result showed that the protective effect of VT was receptor Gb\textsubscript{3} independent in JKT-C cells. A time course experiment was then conducted to determine the minimum period necessary for VT to exert its protective effects (Figure 3.4 b). The result revealed that when cells were treated with VT for shorter durations prior to infection, viral production had a slightly greater decrease compared to the samples treated for longer durations. The 1hour treated sample showed a 74% decrease where the 45hour treated sample only showed a 58% decrease. The effect of treatment plateaued between 1 and 19 hours so for future experiments, JKT-C cells were only treated for 1 hour with verotoxin before HIV-1 infection.
Figure 3.2 – Verotoxin is non-toxic to Jurkat-C cells. Cells were treated with different concentrations of VT1 or VT1B in 10x serial dilutions. Cell proliferation was measured by the REDOX indicator dye alamarBlue®. a) There was a linear correlation between alamarBlue fluorescence intensity and the number of actively proliferating cells in JKT-C and THP-1 cells. b) JKT-C cells were treated with VT1 or VT1B for 3 days and proliferation was calculated as percentage of no VT control. c) JKT-C cell viability with 1µg/mL VT1, VT2, or VT1B treatment was also measured by Trypan Blue dye exclusion assay. d) The Gb3 positive THP-1 cell line was used as a positive control for VT treatment. Error bars represent standard error mean (n=4).
Figure 3.3 – Verotoxin does not affect Jurkat-C global protein synthesis. Jurkat-C cells were treated with 1µg/mL VT1, VT2 or VT1B for 4 days then nascent protein synthesis was measured by $^3$H-Leu incorporation assay. a,b) Total radioactive incorporation was decreased by 7%, 9% and 5% respectively. c) 10% reducing SDS-PAGE gel was used to resolve the proteins and visualized by autoradiography. No significant change in the pattern of global nascent protein synthesis was observed.
Figure 3.4 – Verotoxin treatments of Jurkat-C cells significantly reduce subsequent HIV-1_{IIIB} infection. a) JKT-C cells were treated for 3 days with VT1 or VT2 and the toxins were removed by washing with culture media prior to infection with HIV-1_{IIIB} (m.o.i. = 0.1). Post infection supernatants were collected and HIV-1 viral productive was measured by p24^{gag} ELISA (Zeptometrix). b) A time course experiment was conducted to determine the minimum time required for VT treatment to achieve viral reduction. Error bar represents standard error mean (n = 4).
3.3.1.4 Individual VT1A and VT1B subunit treatments of Jurkat-C cells and HIV-1\textsubscript{IIIb} infection

To determine which subunit of VT was responsible for inhibiting HIV-1\textsubscript{IIIb} infection in JKT-C cells, infection experiments were conducted on cells treated with individual VT1A or VT1B subunit alone. 1\mu g/mL of VT1A subunit or VT1B subunit was added to JKT-C cells for 1 hour and washed away prior to HIV-1\textsubscript{IIIb} infection (m.o.i. = 0.1). The results showed that only VT1A can significantly reduce viral production after infection (Figure 3.5). The efficacy of VT1A subunit was comparable with the VT1 holotoxin (61.8% versus 50% reduction). Even though a small reduction was seen with VT1B treated samples, the result was not statistically significant. This result indicated that VT1A subunit was able to protect JKT-C cells against HIV-1 infection without the involvement of Gb\textsubscript{3}. However, upon repetition in later experiments, it was found that VT treatment of JKT-C cells produced inconsistent infection results. Viral production reduction seen in VT treated JKT-C varied between experiments, even though the VT samples used was confirmed to be active with VT sensitive Vero cells.

3.3.1.5 Cell passage effect of Jurkat-C HIV-1\textsubscript{IIIb} susceptibility

Since previous infection data from JKT-C cell showed considerable variations, a side by side comparision infection was conducted using two batches of JKT-C cells that have been in culture for different durations (Figure 3.6). This infection was repeated twice on two consecutive days with all conditions kept identical. Cells were either treated with 1\mu g/mL of VT1 for 1 hour or left as no VT control. The results showed that JKT-C infectability by HIV-1\textsubscript{IIIb} varied between the two batches of cells. Also the efficacy for the VT protective effect against HIV-1 varied between the two cell batches and between different dates.
Figure 3.5. – HIV-1 inhibitory effect of verotoxin requires catalytic A subunit. Jurkat-C cells were treated with either 1µg/mL of VT1, VT1A or VT1B for 1 hour prior to infection with HIV-1IIIb. Viral production was measured using day 7 infection culture supernatant by p24<sup> gag </sup>ELISA. Error bars represent standard error mean and ** indicates p < 0.03. (n=4)
**Figure 3.6** – Passage number affects HIV-1\textsubscript{IIIB} susceptibility of Jurkat-C cells and effectiveness of VT1 treatment. Two different batches of Jurkat-C cells that were grown for different durations in culture were infected in a side by side comparison experiment with same batch of HIV-1\textsubscript{IIIB} virus. Cells were treated with 1\(\mu\)g/mL VT1 or left as no VT control. The experiment was repeated in two consecutive days. p24\textsuperscript{ gag } ELISA was performed on Day 5 post infection supernatants to measure viral production. Error bar represents standard error mean and statistical significance was indicated by * (n =4, p<0.05).
3.3.2  Anti-HIV-1 effect of VT in activated PBMCs

3.3.2.1  Characterization of PHA/IL-2 activated PBMCs

PHA/IL-2 activated PBMCs are over 95% T-cell blasts (Branch & Mills, 1995). This was confirmed using flow cytometry for the PBMC cell cultures used in this study. Freshly isolated PBMCs were activated with PHA/IL-2 for 4 days. On day 4, cells were stained with anti-CD3, anti-CD14 or anti-CD19 antibody for detection of T cells, monocytes and B cells respectively by flow cytometry. The results (Figure 3.7) showed that over 97% of the cell population was in fact T cells and a small 19% of the cells were positive for CD19. This indicates that the vast majority of the PBMC cell culture is susceptible to the T cell tropic HIV-1_IIIb virus.

Gb3 expression levels in PBMCs were also determined using 14C-galactose metabolic labeling and flow cytometry. Gb3 is the only known receptor for VT, which can trigger the receptor mediated retrograde transport pathway and results in ribosomal inactivation and cell death.

Previous experiments in our lab showed VT treatment produced a significant reduction in PBMC HIV-1 productive infection. It is then important to know whether VT is affecting PBMCs in its classical Gb3 dependent manner. Activated PBMCs were treated with or without VT1 and then metabolically labeled with 14C-galactose for 18 hrs. Glycosphingolipids were extracted, separated on TLC plates and exposed to film (Figure 3.8a). No Gb3 was detected after 4 days of exposure. However, after 3 months of exposure, an additional band appeared corresponding to the position of the Gb3 standard. This species was missing in the VT treated sample, suggesting it was indeed Gb3 and that VT had killed this Gb3 expressing subpopulation in activated PBMCs. This finding was in agreement with previous results, which showed PHA/IL-2 activation induced Gb3 expression in PBMCs (Lund et al., 2006). Gb3 was also detected by flow cytometry by labeling activated PBMCs with Alexa-488 conjugated VT1B (Figure 3.8b). The result showed in control activated PBMCs only 2.83% of the cells were positive for Gb3. The percentage went down to 1.2% after VT1 treatment. These results indicate the VT sensitive population within activated PBMCs is very minute. Therefore VT elimination of this small population would be unlikely to have significant effect on HIV-1 susceptibility of activated PBMCs. Another interesting observation was that not only Gb4 was still present in the VT treated samples, but it also increased comparing to control sample.
Figure 3.7 – Cell surface marker labeling of PHA/IL-2 activated PBMCs. a) PBMCs were activated for 3 days with PHA/IL-2, and then labeled with anti-hCD3, anti-hCD14 or anti-hCD19 antibody for flow cytometry analysis. b) Bar graph summaries the percentage expression of cell surface markers.
Figure 3.8 – Gb₃ expression in PHA/IL-2 activated PBMCs. a) PBMCs were treated with 500ng/mL of VT1 and activated with PHA/IL-2. Control cells had no VT treatment. On day 2, 2 µCi/mL of ¹⁴C-galactose and 0.5µCi/mL of ¹⁴C-Serine were added to the cell culture over night. The cells were then washed once with PBS, subjected to total neutral glycosphingolipid extraction and purified using silica column. Extraction samples were resolute using TLC and exposed to autoradiography film for 4 days and 3 months. Arrows point to location of Gb₃. b) PBMCs were activated and treated with VT1 as before, then stained with Alexa-488-VT1B on day 4. Histograms represent Gb₃ expression profile. Negative gate was set using unlabeled control.
3.3.2.2 Inhibition of HIV-1 infection by VT treatment

Infection experiments were performed with PHA/IL-2 activated PBMCs using a protocol similar to the one used for Jurkat-C cells. The difference being VT is added for the entire duration of activation instead of just 1 hour before infection. Cells were treated with 1µg/mL of VT1, VT1A or VT1B. Both VT1 and VT1A significantly reduced viral production. A reduction was also seen in VT1B treated samples, but was not statistically significant (Figure 3.9a). It was noticed that VT affected cell growth, thus cell number was counted in post infection culture (Figure 3.9d). A 50% proliferation was observed with 1µg/mL VT1 treatment and a 38% proliferation was observed with 1µg/mL VT1A treatment. However, VT1B had no effect on cell viability. It is interesting to note that VT was removed before infection, thus the reduction of cell growth could be initiated in pre-infection culture. Since cell number can affect the amount of viral production, viral production was normalized by dividing p24\(^{\text{gag}}\) value with viable cell count (Figure 3.9b). The general pattern remains the same as pre-adjustment values. On a per unit cell basis, VT1 reduced viral production up to 75% and VT1A reduced viral production up to 85% (Figure 3.9c).

The efficacy of VT1A to inhibit HIV-1\(_{\text{IIIB}}\) productive infection was tested by using 3 different concentrations on PHA/IL-2 activated PBMCs (Figure 3.10 a, b). On a per unit cell basis, both 1µg/mL and 0.1µg/mL of VT1A effectively inhibited HIV-1\(_{\text{IIIB}}\) viral production up to day 7 post infection and a delayed viral production was observed with 0.01µg/mL of VT1A (Figure 3.10 b). VT1A appeared to be 10 times more effective than VT1 holotoxin. VT1B had no effect in terms of blocking viral production. Also it appears that the presence of VT1A subunit, both in the holotoxin and as subunit alone, can significantly inhibit the proliferation of post infection cell culture. A dose dependent growth reduction was observed in response to VT1A treatment (Figure 3.10c). Since VT was removed by multiple washes of the culture both before and after infection, the inhibitory effects must have taken place before infection.
Verotoxin protects PHA/IL-2 activated PBMCs against HIV-1\textsubscript{11IB} infection. Freshly isolated PBMCs were treated with 1\(\mu\)g/mL of VT1, VT1A or VT1B at the same time as PHA/IL-2 activation before infection with HIV-1\textsubscript{11IB} (m.o.i. = 0.3, n = 4). Productive infection were measured by p24\textsubscript{gag} ELISA. a) Total viral production of post infection culture. b) Viral production normalized by cell count. c) Percentage productive infection as compared to control sample. d) Percentage cell proliferation, where viable cells were counted using Trypan blue and calculated as a percentage of control. Asterisks indicate statistical significance compared to control (*: p < 0.05; ** p < 0.03).
**Figure 3.10** – VT1A dose response of HIV-1\textsubscript{IIIB} infection of PHA/IL-2 activated PBMCs. PBMCs were treated with VT1A in 10 fold serial dilutions (1\(\mu\)g/mL, 0.1\(\mu\)g/mL or 0.01\(\mu\)g/mL) during activation. HIV-1\textsubscript{IIIB} infection was conducted 4 days post activation (m.o.i. = 0.3, n = 4). a) Viral production was measured by p24\textsubscript{gag} ELISA for day 3, 5 and 7 infection culture supernatant. Asterisks indicate statistical significance compared to control (*: p < 0.05; **: p < 0.03). b) Viral production over time. c) Viable cell count over time by Trypan blue exclusion. All viral production values were normalized by cell count.
3.3.2.3 Mode of action of VT in protecting PBMC against HIV-1\textsubscript{HIV} infection

3.3.2.3.1 VT inhibits PBMC cell proliferation in a receptor Gb\textsubscript{3} independent manner

Since it was observed that after viral infection the cell proliferation rate of VT treated PHA/IL-2 activated PBMCs are significantly lower than that of control samples, it was necessary to determine if the growth reduction was already occurring during the activation period. The cell proliferation assay was performed by treating the cells with increasing concentrations of VT1 or VT1A. The result revealed that PBMC cell proliferation was reduced as a result of VT treatment in a dose dependent manner (Figure 3.11a). PBMCs were also isolated from four different donors and all showed similar proliferation reductions in response to VT (Figure 3.11b). The LC\textsubscript{50} values were approximately 0.4\mu g/mL (10nM) and 3.4\mu g/mL (40nM) for VT1A and VT1 respectively. To confirm that the VT1A sample used was not contaminated with VT holotoxin, a VT cytotoxicity titration curve was produced using VT sensitive Gb\textsubscript{3} positive THP-1 cells (Figure 3.11c). A 5 log orders difference was observed between the LC\textsubscript{50} of VT1 and LC\textsubscript{50} of VT1A in THP-1 cells. This could correspond to a 1:50,000 contamination ratio of holotoxin within the VT1A preparation. For every 1\mu g of VT1A there could be 20pg of VT1 present and that amount of holotoxin is non-toxic to THP-1 cells. However, the LC\textsubscript{50} values for VT1 and VT1A in PBMCs were only 1 log order apart and the cytotoxicity curves were spread over a much larger concentration range. Therefore VT1 was affecting PBMCs in a different manner than the Gb\textsubscript{3} dependent cytotoxicity of THP-1 cells.

To further eliminate the involvement of VT1B subunit in causing PBMC sensitivity to VT, a VT1B neutralization assay was performed. VT1B antiserum treatment prevents subsequent binding to receptor Gb\textsubscript{3} and stops the cytotoxic effect of VT. The result showed that VT1B neutralization could effectively protect Gb\textsubscript{3} + THP-1 against VT1, but did not prevent cell proliferation reduction in VT1 or VT1A treated PHA/IL-2 activated PBMCs (Figure 3.12). Also VT1A treatment did not affect THP-1 cell proliferation. Therefore VT affected PBMCs in a manner independent of its classical receptor Gb\textsubscript{3}. 
Figure 3.11 - Cell proliferation assay of PBMCs after PHA/IL-2 activation and VT treatment. VT was added to cell culture just after isolation and remains present during PHA/IL-2 activation. Cell proliferation was measured on day 4 using alamarBlue® fluorescent dye indicator assay. a) VT titration curve plotted as percentage proliferation compared to no VT control sample in microgram and nanomolar VT concentrations. b) PBMC samples were collected from 4 different donors and activated in the presence of 1µg/mL VT1, VT1A and VT1B as indicated. c) VT cytotoxicity titration curve using VT sensitive G3+ THP-1 monocytic cell line.
Figure 3.12 - VT1B neutralizing antiserum treatment does not prevent VT growth inhibition of activated PBMCs. PBMCs were activated with PHA/IL-2 for 4 days. a) VT1 or VT1A were pre-incubated with neutralizing anti-VT1B antiserum for 30 minutes at 37°C before adding to activated PBMCs. After 4 days, cell proliferation was measured with AlamarBlue® and percentage proliferation was calculated based on control culture values (no toxin). b) THP-1 a GB3 positive and VT sensitive cell line was used as positive control to demonstrate efficacy of anti-VT1B antiserum protection.
VT does not affect PHA/IL-2 activated PBMC cell population composition

Flow cytometry was performed to determine whether VT treatment changed the cell composition of activated PBMCs. In particular, if there was a reduction in the T cell population and an increase in monocyte or B cell populations. It has been shown that CD4 negative cells, including neutrophils, tonsil-derived B-cells and monocyte derived macrophages, can enhance T-cell susceptibility to HIV-1 (Ancuta et al., 2006; Olinger et al., 2000). PHA/IL-2 activated PBMCs were labeled with antibodies against CD3, CD14 or CD19 for the detection of T-cells, monocytes and B cells respectively. The results are summarized in Table 3.1. Very subtle changes were observed in the percentage of CD3+ T-cells in the cell cultures treated with VT1, VT1A or VT1B compared to control sample. There was a 1.7% decrease in the VT1 treated sample and a 3.2% decrease in the VT1A treated sample. A reduction in the percentage of CD14+ monocytes was seen in both VT1A and VT1B treated samples. Finally, a small increase in the percentage of CD19+ B-cell was detected in all samples, which is opposite from expectation for reducing viral infection. These findings show that the reduction in HIV-1 infection in VT1 and VT1A treated cells was not due to changes in cell population composition.

Another way to inhibit viral infection is by reducing the number of target CD4+ T-cells. Flow cytometry was conducted to determine the ratio of CD4+ T-cells vs. CD8+ T-cell after VT1A treatment in 4 day activated PBMCs. The result showed there was no difference between VT1A treated sample and control sample (Table 3.2). Thus the percentage of HIV-1 susceptible cells remained the same and could not have contributed to the productive infection reduction.
Table 3.1 Cell surface marker labeling of VT treated PHA/IL-2 activated PBMCs.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% CD3 +</th>
<th>% CD14 +</th>
<th>% CD19 +</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>97.4</td>
<td>1.96</td>
<td>18.9</td>
</tr>
<tr>
<td>1µg/mL VT1</td>
<td>95.7</td>
<td>0.62</td>
<td>26.5</td>
</tr>
<tr>
<td>1µg/mL VT1A</td>
<td>94.2</td>
<td>0.35</td>
<td>26.8</td>
</tr>
<tr>
<td>1µg/mL VT1B</td>
<td>97.9</td>
<td>0.30</td>
<td>23.3</td>
</tr>
<tr>
<td>Isotype control</td>
<td>1.73</td>
<td>0.33</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Table 3.2 Effect of VT1A treatment on CD4 positive vs. CD8 positive T cell composition.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% CD3 +</th>
<th>% CD4 +</th>
<th>% CD8 +</th>
<th>CD4 + : CD8 +</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>85.1</td>
<td>41.7</td>
<td>47.0</td>
<td>0.9</td>
</tr>
<tr>
<td>1µg/mL VT1A</td>
<td>88.1</td>
<td>43.5</td>
<td>49.2</td>
<td>0.9</td>
</tr>
</tbody>
</table>
3.3.2.3.3 VT did not affect global phospho-Tyrosine pattern but reduced nascent protein synthesis in PBMCs

Productive HIV-1 infection requires actively proliferating cells (Yoder et al., 2008). T cell activation is achieved by interaction of major histocompatibility complex (MHC) on antigen-presenting cells (APC) with the T cell receptor (TCR). As a result several non-receptor tyrosine kinases are activated resulting in signal transduction leading to proliferation, adhesion and migration. Therefore changes in phospho-Tyr pattern can be used as an indicator for T cell activation state. Furthermore, tyrosine kinase inhibitors have been found to inhibit syncytia formation in Jurkat T cells (Cohen et al., 1992) and HIV-1 entry in primary macrophages (Stantchev et al., 2007), suggesting the importance of Tyr phosphorylation in HIV-1 infection. Western blot was performed to examine phospho-Tyr pattern in activated PBMCs. PBMCs were activated with either PHA/IL-2 or phorbol myristate acetate (PMA). Samples were either treated with VT1A or left as no VT control. Whole cell lysate was run on a 10% reducing SDS-PAGE gel and phosphor-Tyr was detected using 4G10 phospho-Tyr antibody. Equal protein samples (20 µg) were loaded based on BCA protein assay. Results showed no difference in the global phospho-Tyr pattern between control and VT1A treated PBMCs within the same activation condition (Figure 3.13). Most of the Tyr phosphorylations were concentrated in the high molecular weight proteins above 55kDa.

Nascent protein synthesis was also measured in VT treated PBMCs to determine whether the toxin was affecting the cell through its classical protein synthesis inhibition effect. Activated PBMCs were either treated with 1µg/mL of VT1A for the entire duration of activation (4 days) or just for 3 hours on day 4 of activation. Nascent protein synthesis was analyzed using ^3^H-Leu incorporation assay and TCA precipitation. Radioactivity incorporation was measured by beta-counting. The result (Figure 3.14) revealed that VT1A significantly reduced the rate of protein synthesis in PHA/IL-2 activated PBMCs, which was achieved in the absence of its receptor Gb3. It is possible that VT1A gained access into the cytosol of PBMCs and inhibited ribosomal function. The reduced rate of protein synthesis could also be a consequence of cell proliferation reduction.
**Figure 3.13** - VT1A treatment did not alter global phospho-Tyr pattern in activated PBMCs. Isolated PBMCs were activated with PHA/IL-2 or PMA. On day 4, cells were lysed and run on a 10% reducing SDS-PAGE gel. Each lane contains 20µg of total protein. Phospho-Tyr was detected using 4G10 antibody. The same membrane was stripped and re-probed with anti-tubulin antibody for loading control.
**Figure 3.14** – Protein synthesis inhibition of VT1A treated activated PBMC. PBMCs were activated with PHA/IL-2 for 4 days then subjected to $^3$H-Leu incorporation assay and TCA precipitation. Nascent protein synthesis was quantified by beta counting. Samples were treated with 1µg/mL VT1A either for the duration of activation (4 days) or for 3hrs just prior to radioactive labeling. (**: p = 0.027, n = 3)
3.3.2.3.4 Gene expression array analysis of VT1A treated PHA/IL-2 activated PBMCs

To further examine the effects of VT treatment on normal cell function of PBMCs, gene expression array analysis was conducted. Isolated PBMCs were activated with PHA/IL-2 for 4 days. Test samples were treated with 1µg/mL of VT1A at the time of activation and control samples were treated with vehicle only (n = 3). Total RNA was extracted using TRIzol® reagent and sent for gene expression array analysis using Human WG-6 Expression BeadChip. Array results were analyzed using the LIMMA algorithm. Genes with an adjusted p value of < 0.03 were considered differentially expressed. Figure 3.15 shows the heatmap of gene/probe sets with p value of < 0.1 (see Appendix for more detailed list). In general, the effect of VT1A on PBMC gene expression was highly specific since only 49 genes/probes were significantly affected out of a total of 36,604 genes/probes. 30 genes/probes were up-regulated (0.082%) and 19 genes/probes were down-regulated (0.051%) (Appendix Table 1). The most striking finding was that 10 of the upregulated genes belonged to histone cluster proteins, which also made up the top 2 ranking genes. This has led to the speculation that the anti-HIV-1 effect of VT might be exerted through increased histone expression in PBMCs. Inhibition could be achieved in two ways: by preventing viral integration into the host cell genome and by preventing viral gene expression.

Suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor, was used to investigate the role of histone in preventing HIV-1 infection in PBMCs. HDAC inhibitor prevents histone deacetylation and leads to dissociation of histone from DNA. In particular, the acetylation of a specific nucleosome in the 5’ LTR of HIV-1 provirus can break the latency of infection. SAHA treatment is expected to promote the open chromatin structure, thus enhances HIV-1 provirus incorporation and expression. This should counteract the effect of a possible increase in chromatin histone content due to VT1A treatment. In addition, SAHA could also increase viral production by preventing latency. To test the possibility that histone upregulation is a cause of reduced viral production, PHA/IL-2 activated PBMCs were treated with 0.5µg/mL of VT1A with or without 2.5µM of SAHA. The treatment was added either at the time of activation or on day 3 post-activation. Cells were infected with HIV-1<sub>M</sub> on day 4 (m.o.i = 0.3, n = 4). The result showed that even though SAHA treatment significantly reduced viral production, it severely inhibited PBMC proliferation (Figure 3.16). Therefore, it was still unclear whether histone is involved in the anti-HIV-1 effect of VT1.
Figure 3.15 – HeatMap of differentially expressed genes in PBMCs after VT1A treatment. Genes/probes with an adjusted P value of <0.1 are shown. C: control samples with no VT treatment. T: samples treated with 1μg/mL VT1A for 4 days during PHA/IL-2 activation. Expression levels are represented by color, with green being low expression and red being high expression. (See Appendix for a list of gene descriptions, fold change and adjusted p values)
Figure 3.16 – Effect of VT1A and SAHA treatment on PHA/IL-2 activated PBMC HIV-1\textsubscript{IIIB} infection. Freshly isolated PBMCs were activated by PHA/IL-2. Samples were treated with VT1A (0.5\textmu g/mL) and/or SAHA (2.5\textmu M) at the time of activation or 1 day prior to infection (late). On day 4, cells were infected with X4 HIV-1\textsubscript{IIIB} virus. Infection supernatants were collected on day 5 of infection and HIV-1 production was quantified by p24\textsuperscript{gag} ELISA. Viable cells were counted under Trypan Blue and calculated as a percentage of control sample cell count. Error bars represent standard error mean (n = 4).
3.4 Discussion

The results are in support of the hypothesis that VT can serve as an inhibitor of productive X4 HIV-1\textsubscript{IIB} infection. Significant viral production reduction was observed after VT treatment in both JKT-C cell line and primary PHA/IL-2 activated PBMCs. In both cases, VT1A subunit also showed comparable inhibition effect on viral production and in fact appeared to be more potent than the holotoxin (61.8% versus 50% reduction in JKT-C and 85% versus 75% reduction in PBMCs) (Figure 3.5 and Figure 3.9).

The T-cell leukemia cell line JKT-C was used to study the effect of VT to prevent X4 HIV-1\textsubscript{IIB} infection in the absence of its receptor Gb\textsubscript{3}. The JKT-C cell line was verified to be unable to express Gb\textsubscript{3} and that VT treatment did not affect cell proliferation or protein synthesis. Fluorescence labeling was unable to detect any bound VT on the cell surface (data not shown). Thus, the anti-HIV-1 effect of VT appears to be independent of its classical receptor mediated retrograde transport pathway and ribosomal inactivation effect. Furthermore, any VT present in the media was washed away prior to infection, yet HIV-1 infection was significantly reduced after the treatment. Thus, any effect that VT had on JKT-C cells must have already taken place prior to infection. The effect of VT seems to be a rapid process since only 1 hour of exposure was needed to produce the inhibitory effect. Finally, the fact that VT1A subunit only can protect against HIV-1 as effectively as the holotoxin further confirms the independence of Gb\textsubscript{3}.

Variable infection results were observed in later experiments, which could be a consequence of cell passage (Figure 3.6). In the infection experiment, viral infection was quantified by the amount of viral p24 produced. It has been found that different clones and different passage numbers of JKT-C cell cultures have different ability to support HIV-1 viral production after infection (Cervantes-Acosta et al., 2001). The difference seen in viral production could be more than the result of altered viral susceptibility. Therefore, the JKT-C cell line is a less than perfect cell model for HIV-1 infection. Therefore it was necessary to conduct further infection studies with primary human PBMCs.

In the case of primary PHA/IL-2 activated PBMCs, the consistent reduction of viral production after VT treatment could be caused by four possible mechanisms. First, VT treatment led to significant reduction in the proliferation of activated PBMCs (Figure 3.11). The number of proliferating cells was measured using alamarBlue\textsuperscript{®} dye which detects the reductive
environment created by cell metabolic activity. Viral production appeared to be proportional to the amount of cell proliferation and was dependent on the concentration of VT1A treatment (Figure 3.10). Similar reduction of metabolic activity was seen in VT1 treated PHA stimulated bovine PBMCs, where the reduction was not caused by apoptosis but that VT abolished the proliferation stimulatory effect of the mitogen (Menge et al., 1999). Thus VT reduces HIV-1 viral production by reducing cell activation, where cell activation is a requirement for viral replication (Rosenberg & Fauci, 1991; Yoder et al., 2008). Second, microarray analysis in our study revealed that VT1A treatment significantly up-regulated genes that are pro-inflammatory and induce T cell activation. For instance, the expression of DNA damage inducible GADD45β was increased by 3.43 fold, which is involved in T cell apoptosis and also important for production of inflammatory cytokine such as interferon γ (Chi et al., 2004; Lu et al., 2004). To a lesser extent the STAT5 inhibitor CISH was also upregulated by 2.23 fold. CISH is involved in protein kinase C activation in T cells using TCR signaling cascade (Chen et al., 2003). Together with colony stimulation factor 2, whose expression was increased by 3.63 fold, CISH could induce hyperproliferation as implicated in an acute myelogenous leukemia study (Hunter et al., 2004). This T cell activation state could potentially help eliminate initial virus infection similar to the asymptomatic phase of HIV infection where host immune response keeps blood viremia low (McMichael et al., 2010) at the same time replenish itself by increased turnover (McCune et al., 2000). Third, VT1A treatment significantly inhibited nascent protein synthesis in PBMCs (Figure 3.14). This could be the cause of reduced cell proliferation and indicated that VT was acting through its classical ribosomal inactivating effect thus rendering infected cells unable to synthesize viral proteins for viral progeny. Finally, VT1A treatment up-regulated histone cluster protein expression as shown in microarray analysis, which could result in increased histone content in the chromatin and a more closed chromatin structure. This closed and more transcriptionally inactive chromatin structure could hinder productive infection since HIV-1 proviral cDNA preferentially inserts into transcriptionally active genes for enhanced expression (Brady et al., 2009; Jordan et al., 2001; Schroder et al., 2002).

The possibility that increased histone expression after VT1A treatment is the reason behind reduced HIV-1 productive infection in PBMCs was examined by treating the cells with the HDAC inhibitor SAHA. The acetylation state of a specific nucleosome located in the U3 region in the 5’LTR of the integrated HIV-1 genome is directly related to viral transcriptional activation
SAHA is a FDA approved drug to treat lymphocytic leukemia (Duvic & Vu, 2007). In recent years it has been studied \textit{in vitro} to help break HIV-1 viral latency and promote viral eradication (Archin et al., 2009; Demoonte et al., 2004; Edelstein et al., 2009). SAHA promotes histone acetylation and thus results in increased HIV-1 gene expression, which may counteract the effect of VT1A treatment in PBMCs. However, SAHA treatment was found to be severely inhibitory for PBMCs proliferation (Figure 3.16). This effect could be due to the antineoplastic property of SAHA. Thus it is still undetermined whether VT inhibited productive HIV-1 infection through increased histone expression.

The mode of action of VT was determined to be independent of its receptor Gb3 in Jurkat cells and primary PHA/IL-2 activated PBMCs. Only 3% of the PBMCs were positive for Gb3 and the VT1A subunit alone without the receptor binding subunit exhibited similar anti-HIV-1 effect as the holotoxin. The fact that VT1A caused protein synthesis inhibition suggests that it can gain access into the cytosol. However, the mechanism is still unknown. It was speculated that VT1 and VT1A could be taken into the cells randomly through macropinocytosis. The random nature of the uptake event requires much higher concentrations for VT to take effect in PBMCs as indicated by the LC$_{50}$ values in relation to that of the Gb3 positive THP-1 cells. In receptor mediated endocytosis of VT, the vast majority of the toxin remains inside the ER and only 4% of VT1A is translocated to the cytosol (Tam & Lingwood, 2007). Only one molecule of VT1A in the cytosol is required to cause cell death. Some protein synthesis inhibition still occurs when retrograde transport is inhibited in vero cells and when the majority of the toxin is still concentrated in endosomes. This suggests alternative routes for VT to escape into the cytosol without the involvement of Golgi/ER. In addition, the reduction of the proteolytically cleaved VT1A subunit for activation is considered to be the rate limiting step in the cytosol translocation process. Since the VT1A subunit preparation was already fully activated, it was more potent compared to the holotoxin in inhibiting cell proliferation.
Chapter 4: Relationship between Gb₃ expression and HIV-1 susceptibility: Verotoxin selection of THP-1 cells

4.1 Summary

To further investigate the role of Gb₃ and VT in HIV-1 infection, a subpopulation of THP-1 cells with low Gb₃ expression was successfully selected from wild type Gb₃ positive THP-1 cells using VT1. The selected THP-1 cells were confirmed to have greatly reduced total and cell surface Gb₃ expression levels and were shown to be 60 times more resistant to VT1 and 1,000 times more resistant to VT2 compared to wild type cells. Interestingly, the selected THP-1 cells were found to be completely resistant to X4 HIV-1[subscript IIIb] infection. The p24 production level of selected THP-1 cells was below the detection limit of the ELISA used. This finding was unexpected since previous studies from our lab indicated Gb₃ as a natural resistance factor for HIV-1 infection. Epifluorescence microscopy and flow cytometry revealed that VT1 selected THP-1 cells had reduced levels of the cell surface X4 HIV-1 co-receptor CXCR4 compared to wild type cells. The MFI value for CXCR4 was reduced by 24% to 64% depending on the antibody used. This could be one explanation for their resistance to HIV-1[subscript IIIb]. VT1B binding to receptor Gb₃ was found to have no effect on the availability of CXCR4 for antibody detection, indicating they were not in close physical contact. Also in the literature, CXCR4 was not found in close association with Gb₃ enriched lipid rafts before HIV-1 infection (Kozak et al., 2002). Furthermore, since VT1 selected THP-1 cells were still nearly 100% positive for CXCR4 yet became completely resistant to HIV-1[subscript IIIb], CXCR4 reduction could not be the only factor affecting THP-1 viral infection. These experiments indicated that the conditions required for HIV-1 infection may be more complex and manipulation of Gb₃ levels could bring other unexpected changes in cell function which could affect HIV-1 susceptibility.

4.2 Background

During HIV-1 infection the viral envelope glycoprotein gp120 binds with the host cell receptor CD4 and chemokine coreceptor CCR5 or CXCR4 for viral host cell fusion (Alkhatib et al., 1996; Dalglish et al., 1984; Feng et al., 1996). In addition to these classical HIV-1 infection receptors many glycosphingolipids (GSLs) are found to be involved in the fusion process (Bhat et al., 1992; Delezay et al., 1996; Viard et al., 2004). In particular globotriaosyl ceramide (Gb₃) is found to be recognized by gp120 and there has been some debate on the role of Gb₃ in HIV-1
infection. The works done by Fantini et al and Puri et al (Fantini et al., 2000; Puri et al., 1999) have suggested that Gb₃ promotes infection by recruiting HIV-1 receptors to the infection site. However, studies performed in Dr Branch’s lab and our lab have found that Gb₃ serves as a natural resistance factor for HIV-1. Genetic study found PBMCs from the p blood group phenotype, which express no Gb₃, were much more susceptible to HIV-1 infection compared to normal PBMCs (Lund et al., 2009). PBMCs from Fabry disease patient, which has an accumulation of Gb₃, were resistant to HIV-1 infection (Lund et al., 2005). Pharmacological manipulation study using P4 (inhibitor of GlcCer, and hence Gb₃, biosynthesis) or DGJ (prevents Gb₃ breakdown) treated human monocytic THP-1 cells also revealed a negative correlation between Gb₃ levels and HIV-1 susceptibility (Ramkumar et al., 2009). Furthermore, a soluble mimic of Gb₃ has been shown to inhibit HIV-1 viral envelope fusion with target cell membrane in Jurkat cells and PHA/IL-2 activated PBMCs (Lund et al., 2006).

In this study, the role of Gb₃ in HIV-1 susceptibility was further investigated by selecting a subpopulation within wild type THP-1 cells with reduced Gb₃ expression levels. The human acute monocytic leukemia cell line (THP-1) is Gb₃ positive (Kniep et al., 1985) and very sensitive to verotoxin. Previous pharmacological manipulation study used P4 treatment to eliminate Gb₃ expression (Ramkumar et al., 2009). However, this drug inhibits the synthesis of all GlcCer-based glycosphingolipids, which could severely affect many cellular functions. Factors other than Gb₃ may be involved in the increased HIV-1 susceptibility. In the current study, THP-1 cells were treated with increasing concentrations of VT1, and the condition where only 10% of the cells survived was used to select a Gb₃ low population. This method exploits the natural variation of Gb₃ levels within the cell line and is a more natural way to eliminate Gb₃ compared to treatments with P4 in the previous study.

4.3 Results
4.3.1 Selection and characterization of VT-1 resistant THP-1 cells
To select for THP-1 cells with low Gb₃ expression, wild type THP-1 cells were incubated with VT1 in 10x serial dilutions. Cell growth was monitored for 6 days and proliferation was measuring using alamarBlue® on day 4 of treatment (Figure 4.1). The LC₅₀ of VT1 for wild type THP-1 cells was measured to be 50fg/mL. Samples treated with 1pg/mL and 10 pg/mL of VT1 had a cell survival rate of approximately 10%. These samples were then collected on day 6,
pooled together into fresh complete RPMI1640 media without toxin and kept growing at 37°C with 5% CO₂. After approximately 4 weeks, the surviving cells were found to be actively proliferating. Another treatment with 1pg/mL VT1 was included to ensure selected cells have maintained their resistance to VT1. Finally VT1 was removed by washing with RPMI media and the resulting cells were named selected THP-1 (selTHP-1). A double selected THP-1 cell line (d. selTHP-1) was also generated from selTHP-1 cells by treating them again with 10x serial dilutions of VT1. After 4 days, the sample with 10% cell survival rate (1µg/mL VT1treated) was collected and allowed to expand in fresh complete RPMI1640 media.

After VT1 selected THP-1 cell lines had been successfully established, they were characterized for VT resistance and Gb₃ expression. VT resistance was measured using a cytotoxicity assay where cells were treated with 10x serial dilutions of VT1, VT1A, VT1B or VT2 (Figure 4.2). VT1 selected THP-1 cells were found to be 60 times and 100 times more resistant to VT1 and VT2 than wild type THP-1 cells respectively. Double selected THP-1 cells were found to be more than 2000 times more resistant to both VT1 and VT2 comparing to wild type cells. It was also noticed that VT1A exhibited cytotoxicity to WT THP-1 and selected THP-1 cells starting from 2ng/mL and 20ng/mL respectively. This could be due to the potential 1:50,000 holotoxin contamination as mentioned in previous section. Finally, wild type THP-1 cells were found to have become less susceptible to VT1 over time based on LC₅₀ values (Figure 4.1b vs. Figure 4.2a). This change could be due to the passage number of cell culture. The Gb₃ expression levels of selected THP-1 cells were much lower comparing to wild type THP-1 cells which was in agreement with VT cytotoxicity result. Total neutral glycosphingolipid extraction and VT overlay have shown there was a 56.6% reduction in total Gb₃ level in selTHP-1 and an 83.5% reduction in d. selTHP-1 cells (Figure 4.3). Other glycosphingolipids including GlcCer, LacCer and Gb₄ were not affected; except an increase in LacCer level in d.selTHP-1 cells.

To test whether reduction in total Gb₃ expression levels in selTHP-1 cells is reflected at the cell surface, epifluorescence microscopy and flow cytometry was performed. THP-1 cells were labeled on ice with Alexa488-VT1B for surface Gb₃ labeling. Figure 4.4a shows epifluorescence microscopy images of a representative population of each THP-1 cell type taken with constant exposure time. Wild type THP-1 cells showed high expression levels of Gb₃, but the staining intensities vary greatly within the sample. selTHP-1 cells had greatly reduced Gb₃ labeling intensity and double selTHP-1 had minimal staining. Figure 4.4b shows the flow cytometry
analysis result of wild type and selTHP-1 cells. The forward scatter and side scatter plot showed selTHP-1 cells had greater side scatter intensity comparing to wild type THP-1 cells, which indicates greater granularity within the selected THP-1 cells. A histogram of Alexa-488-VT1B labeling showed the selected THP-1 cells were a mixed population with variable Gb3 expression levels, whereas the wild type THP-1 cells had much more uniform Gb3 expression. Mean fluorescence intensity (MFI) of selected THP-1 cells was reduced by 71.4% comparing to wild type cells.

4.3.2 Effect of VT1 selection on susceptibility of THP-1 cells to HIV-1\textsubscript{IIIB} infection

Infection experiments were conducted to determine whether selTHP-1 cells have increased susceptibility to HIV-1 infection. Previous genetic and pharmacological studies in our lab have shown a negative correlation between Gb3 expression level and HIV-1 susceptibility (Lund et al., 2009; Ramkumar et al., 2009). A soluble mimic of Gb3 has also been shown to inhibit HIV-1 viral envelope fusion with target cell membrane (Lund et al., 2006). Since selTHP-1 cells have much lower Gb3 expression, it was expected that they would be more susceptible to HIV-1. Surprisingly, the result indicated that selTHP-1 cells were completely resistant to HIV-1\textsubscript{IIIB} infection (Figure 4.5). When p24\textsuperscript{gag} concentration of the post-infection supernatant of selTHP-1 was measured, the OD\textsubscript{450} values fell below the detection range of the p24 capture ELISA. This result suggests there could be factors other than Gb3 expression affecting HIV-1 infection in THP-1 cells.
Figure 4.1 – VT1 cytotoxicity selection of Gb3 positive THP-1 cells. Wild type THP-1 cells were treated with 10-fold serial dilutions of VT1. a) Cells were kept in culture and monitored for 6 days. b) Proliferation was measured using alamarBlue® on day 4 and calculated as a percentage of control culture. On day 6, samples treated with 1pg/mL and 10pg/mL of VT1 were pooled together and kept growing in regular complete RPMI1640 media with no toxin.
Figure 4.2 – VT selected THP-1 cells are more resistant to verotoxins. Wild type (Gb$_3^+$), VT1 selected and double selected THP-1 cells were incubated for 4 days with different concentrations of VT1, VT1A, VT1B or VT2 in 10x serial dilutions. Cell proliferation was measured by fluorescence using alamarBlue®. (Excitation: 544nm, emission: 590nm)
Figure 4.3 – VT1 selected THP-1 cells have greatly reduced total Gb₃ expression. Wild type (WT), VT1 selected (sel) and double selected (d. sel) THP-1 cells were subjected to total neutral glycosphingolipid extraction. Glycosphingolipids were then resolved using TLC. Each lane represents total neutral glycosphingolipids from 1x10⁶ cells. Glycosphingolipid bands were visualized using orcinol spray (a) and Gb₃ was detected by VT-overlay (b). Band density of the VT-overlay plate was measured using the ImageJ program and shown in bar graph (c).
**Figure 4.4a** – VT1 selected THP-1 cells have reduced surface Gb$_3$ expression. THP-1 cells were labeled with Alexa-488 – VT1B (Green) for Gb$_3$ and DAPI (Blue) for nuclei. Images were captured using epifluorescence microscopy. Exposure time was kept constant for all images. A typical field is shown here for each cell type.
**Figure 4.4b** – VT1 selected THP-1 cells have reduced surface Gb$_3$ expression. THP-1 cells were labeled with Alexa-488 conjugated VT1B for Gb$_3$ staining and analyzed by flow cytometry. Live cells were gated in the forward and side scatter plot (P1, red). Histogram represents Gb$_3$ staining profile with Alexa-488-VT1B within P1 gate. Mean fluorescence intensity (MFI) were measured by FACSDeva® and shown in the bar graph.
Figure 4.5 – HIV-1_{IIIb} infection of VT1 selected THP-1 cells. WT and VT1 selected THP-1 cells were infected with HIV-1_{IIIb} (m.o.i = 0.4, n=6). Post-infection supernatants were collected on day 5 and 7 and analyzed using a p24_{gag} ELISA assay (ZeptoMetrix™). Bars represent absorbance readings for p24 detection at 450nm (OD450). selTHP-1 p24 concentrations were unavailable because OD values fall below the standard curve range. WT THP-1 p24 concentrations were 188pg/mL on day 5 and 243pg/mL on day 7. Day 7 samples were diluted 1/2 with culture media for ELISA to ensure WT sample value stay within standard curve range. (**: p < 0.03)
4.3.3 Evaluation of HIV-1 receptor expression in HIV-1<sub>HIB</sub> resistant VT1 selected THP-1 cells

To investigate the cause of reduced susceptibility to HIV-1 observed in VT1 selected THP-1 cells, the expression level of HIV-1 receptor CD4 and co-receptor CXCR4 was measured. Cell surface CD4 expression was determined using flow cytometry. The result showed the amount of CD4 was similar between WT THP-1 and selTHP-1 cells, where nearly 100% of the cells were CD4 positive (Figure 4.6). The MFI value was increased from 92.97 to 146.96 between WT cells and selTHP-1 cells, which appears to be contradictory to the infection result. Total CXCR4 expression in wild type and VT1 selected THP-1 cells were determined by western blot (Figure 4.7). Total cell lysate was obtained and protein concentration was measured by Bio-Rad protein assay. 20µg, 10µg and 5µg of total protein were loaded on a 10% reducing SDS-PAGE gel for each cell type. CXCR4 (MW: 45kDa) was detected using anti-hCXCR4 clone 12G5 antibody and beta-tubulin was used as loading control. The result showed there were comparable amounts of CXCR4 in wild type and selected THP-1 whole cell lysate. Multiple bands were seen on the western blot for CXCR4. This phenomenon has been reported in the literature for multiple CXCR4 expressing cell lines including THP-1, which the author attributed to different glycosylation isoforms of CXCR4 (Sloane et al., 2005).

Epifluorescence microscopy and flow cytometry were performed to detect cell surface expression of CXCR4 in wild type and selTHP-1 cells. Cells were double labeled with TexasRed conjugated VT1B and mouse anti-hCXCR4 antibody clone MAB171 followed by Alexa-488 goat anti mouse antibody. Several images were taken under constant exposure time using epifluorescence microscopy for each cell type. Figure 4.8 shows representative images. The number of cells expressing high Gb<sub>3</sub> or CXCR4 were counted and calculated as a percentage of the total number of cells observed (WT: n = 68; selected: n = 70). The results showed that selTHP-1 cells had a much lower percentages of cells with high Gb<sub>3</sub> expression and the general staining of these cells was weaker than wild type cells. It was also observed that CXCR4 staining was much weaker in selTHP-1 cells, although a few brightly stained cells still existed. A reduction was also observed in the percentage of cells that were high in both Gb<sub>3</sub> and CXCR4. However the difference was not as dramatic as for the individual markers. Flow cytometry analysis using a panel of three different antibodies also confirmed the finding that VT1 selected THP-1 cells had reduced surface CXCR4 expression levels (Figure 4.9). Two of the antibodies
(MAB173 and 12G5) are recommended for flow cytometry by the manufacturer. The pattern of staining for the three antibodies was very similar. The percentage of cells expressing CXCR4 was greater than 99%, except for selTHP-1 stained with MAB173 antibody (94.5%). The MFI values were measured by FACSDeva® program. A decrease of the MFI value for selTHP-1 CXCR4 staining from WT cells was observed with all three antibodies even though the staining intensity of different antibodies was variable. Therefore, these results lead to the speculation that the resistance for X4 tropic HIV-1IIIb infection observed in selTHP-1 could be partially resulted from decreased cell surface CXCR4 expression.

4.3.4 CXCR4 is not in close association with Gb₃.

Flow cytometry was performed to determine if CXCR4 is in close physical association with Gb₃ on cell surface. Glycosphingolipids including Gb₃ are greatly enriched in detergent resistant membranes (DRM)/lipid rafts on cell surface (Katagiri et al., 1999; Thomas et al., 2004). DRM is a “hot spot” for many cell surface receptors. CXCR4 could also be present in Gb₃ enriched DRMs, thus reduced CXCR4 cell surface expression could be a consequence of lack of Gb₃ expression. To determine whether CXCR4 is in close contact with Gb₃, wild type and selTHP-1 cells were incubated with unlabeled VT1B (0.3µg per 0.5x10⁶ cells in 100µL) for 1 hour on ice before adding anti-CXCR4 antibody on ice. If CXCR4 is within Gb₃ rich domains, the bulk of the bound VT1B would interfere with CXCR4 antibody binding, thus reduction in CXCR4 staining intensity would be expected. The result (Figure 4.10) showed there was no difference in CXCR4 staining profile between control sample and VT1B treated sample. This suggested that it is unlikely for CXCR4 to be located in the proximity of Gb₃ in THP-1 cells.
Figure 4.6 – Cell surface CD4 expression of THP-1 cells. WT and selTHP-1 cells were stained with APC-anti-hCD4 antibody. The staining results were measured using flow cytometry. Negative gate was created using no-antibody control. MFI value was calculated using the CellQuest® program.
**Figure 4.7** – Total CXCR4 expression of THP-1 cells. Total cell lysate of wild type and VT1 selected THP-1 cells was separated on a 10% reducing SDS-PAGE gel for western blot analysis. Lanes were loaded with 20μg, 10μg or 5μg of total protein as indicated. Upper panel shows blot stained with clone 12G5 anti-hCXCR4 antibody. Lower panel shows blot stained with anti-beta-tubulin (clone E7) antibody as loading control. (CXCR4 expected MW: 45kDa)
**Figure 4.8** – Cell surface Gb3 and CXCR4 expression of THP-1 cells. Wild type and selTHP-1 cells were labeled with Texas Red conjugated VT1B (Red) for Gb3 and MAB171 (Green) antibody for CXCR4. Cells were viewed using epifluorescence microscopy and images were recorded with constant exposure time. Representative images are shown here for each cell type. Arrow head points to cells with high intensity staining. Bar graph shows the percentage of cells with high surface receptor staining.
Figure 4.9 – VT1 selected THP-1 cells have reduced cell surface CXCR4 expression. Wild type and VT1 selected THP-1 cells were labeled with three different antibodies for CXCR4 (MAB173, MAB171, 12G5) followed by staining with Alexa-488 conjugated goat anti mouse antibody. The labeling profile was detected using flow cytometry. The negative gate was created using secondary antibody only control. CXCR4 staining intensity profiles were shown in the histograms and MFI values were plotted in the bar graph.
**Figure 4.10** – VT1B competition for CXCR4 detection. WT THP-1 and selTHP-1 were pre-incubated with VT1B for 1 hour on ice (0.3µg per 0.5x10^6 cells in 100µL) before staining with anti-CXCR4 antibody (MAB171). Control samples were stained directly with anti-CXCR4 antibody. Negative gate was created based on secondary antibody only control. Histogram shows CXCR4 staining profile. Bar graph shows mean fluorescence intensity.
4.4 Discussion

In addition to the classical HIV-1 receptor CD4 and co-receptor CCR5/CXCR4, many glycosphingolipids have been found to be involved in HIV-1 infection and may function as alternative or auxiliary receptor. The GSLs LacCer, GM3 and GD3 have been found to be able to bind with SPC3, a synthetic peptide for the variable V3 loops of the HIV-1 gp120 (Delezay et al., 1996). GalCer has been purposed as an alternative receptor for HIV in CD4 negative cells (Cook et al., 1994). GalCer and GM3 were found to allow the insertion of gp120 into GSL monolayers as indicated by increased surface pressure (Hammache et al., 1998). Also a soluble mimic of Gb3, adamantylGb3, has enhanced affinity for gp120 compared to Gb3 (Mahfoud et al., 2002).

Recent studies in our lab have found Gb3 to be a potential natural resistance factor for HIV-1 infection. Infection experiments using PBMCs from individuals that naturally express no Gb3, p phenotype, have found them to be hyper sensitive to HIV-1 infection, whereas PBMCs from individuals who over express Gb3, the P1k and P2k phenotypes, were much more resistant to HIV infection compared to wild type PBMCs (Lund et al., 2009). In addition, PBMCs from Fabry disease patients, which have high accumulation of Gb3, were non-susceptible to HIV-1 infection (Lund et al., 2005). However, the reduction in infection was attributed to cell surface CCR5 reduction. Pharmacological manipulation of Gb3 expression levels using the α-galactosidase A inhibitor DGJ and glucosylceramide synthase inhibitor P4 in THP-1 cells and HeLa cells also suggested a negative correlation between Gb3 content and HIV-1 productive infection. However the works done by the group of Fantini and Puri have shown opposing results (Fantini et al., 2000; Hug et al., 2000; Puri et al., 1998). Puri et al have found inhibition of GSL synthesis inhibited HIV gp120 mediated fusion, which can then be reconstituted with reintroduction of Gb3. Fantini et al then purposed that Gb3 present in lipid rafts plays a supportive role in HIV infection by stabilizing the gp120 - CD4 complex and facilitating migration to chemokine co-receptor. However, these works lack real infection data.

In our present study, the role of Gb3 in relation to HIV-1 infection was further addressed by exploiting the natural variation of Gb3 expression in THP-1 cells. A subpopulation of THP-1 with low Gb3 content was selected using VT1. The majority of the cells died during the VT1 treatment and only approximately 10% of the cells survived (Figure 4.1). The resulting selTHP-
1 cells were much more resistant to subsequent VT1 challenges compared to WT THP-1 and have much reduced total and cells surface Gb3 expression (Figure 4.2 - 4.4). However, when selTHP-1 cells were infected with HIV-1\textsubscript{IIIb} virus, they were surprisingly non-susceptible to the virus (Figure 4.5). This result appears to be opposite to previous theory in our lab that Gb\textsubscript{3} serves as a natural inhibitor for HIV-1 infection. It is still not clear how this complete inhibition of viral infection was achieved. However, the presence of Gb\textsubscript{3} in lipid rafts or detergent resistant domains could be a contributing factor, since it plays a role for both HIV-1 infection and verotoxin cytotoxicity. Lipid rafts are specialized domains in the plasma membrane that are enriched in cholesterol and GSLs (Brown, 1998; Simons & Ikonen, 1997). Lateral sorting of lipid species in lipid bilayer according to their affinity for cholesterol produces a L\textsubscript{o} phase enriched in GSLs and a L\textsubscript{d} phase enriched in phospholipids. The L\textsubscript{o} phase exists as small dynamic domains called lipid rafts in living cells, which are also enriched in membrane proteins that are critical in signal transduction. HIV-1 viral fusion with the host cell membrane is highly dependent on the presence of rafts (Manes et al., 2000). Disruption of raft structure by cholesterol extraction using β-cyclodextrin renders cells resistant to HIV-1 infection (Liao et al., 2001). The budding of viral progeny also occurs at site of lipid rafts (Nguyen & Hildreth, 2000). On the other hand, localization of Gb\textsubscript{3} into lipid rafts is correlated to VT sensitivity (Falguieres et al., 2001; Mori et al., 2000) and the density of Gb\textsubscript{3} in lipid raft was also found to be important for VT binding (Hanashima et al., 2008). Taken both effects into consideration, it is possible that by selecting cells with VT1, the fraction of THP-1 cells with high Gb\textsubscript{3} content inside lipid rafts has been eliminated, thus resulting in HIV-1 infection inhibition.

Upon further investigation, it was found that the cell surface CXCR4 expression in selTHP-1 was significantly reduced compared to WT cells, even though the total CXCR4 repression was unaffected. The MFI reduction of surface CXCR4, depending on the detection antibody used varied from 65.3 % to 23.9% (Figure 4.9). It was not clear whether this reduction in surface CXCR4 was a direct result from low Gb\textsubscript{3} expression in selTHP-1 cells, since VT1B competitive binding experiment suggested that Gb\textsubscript{3} and CXCR4 were not in close physical association on the cell surface. This observation is similar to that seen in Fabry patient PBMCs, where the cells have low surface CCR5 expression (Lund et al., 2005). However, internal Gb\textsubscript{3} accumulation, instead of reduction, was speculated to be a possible cause for impaired CCR5 trafficking to plasma membrane. CXCR4 is the co-receptor required for the X4 tropic HIV-1\textsubscript{IIIb} infection, this
could be an explanation for enhance viral resistance. However, other factors must be at play, since nearly 100% of the selTHP-1 cells were still CXCR4 positive. In literature, it has been found that prior to HIV-1 infection the majority of CXCR4 is found outside the lipid raft domain in PBMCs and T cells (Kamiyama et al., 2009; Kozak et al., 2002). However, HIV-1 viral entry requires the relocation of CXCR4 into rafts after gp120 binding in CD4-dependent, X4 tropic viral infection (Kamiyama et al., 2009). It is possible that these low Gb3 expressing selTHP-1 cells have disrupted lipid microdomains that do not allow sufficient CXCR4 raft redistribution, thus preventing infection from occurring. An increase in the surface expression of CD4 in selTHP-1 cells was also observed, where the MFI value increased by 58.1% (Figure 4.6). However, increase in CD4 expression does not necessarily confer enhanced HIV infection, because raft localization is required for CD4 receptor function (Del Real et al., 2002). Since selTHP-1 cells have decreased Gb3, it is possible these cells have altered raft structures, and the extra surface CD4 may not reside within raft domains. Further experiments in characterizing selTHP-1 raft composition are required to understand their HIV-1 resistance.
Chapter 5: General Discussion and Future Directions

5.1 Summary

AIDS, caused by HIV-1 remains one of the most severe pandemics in the world since its discovery in 1983. Despite intensive research efforts over the years, there is still no cure or effective vaccine for this deadly disease. Recent discovery made in our lab have identified the glycolipid globotriaosylceramide Gb$_3$, also known as the P$^k$ blood group antigen, as a natural resistance factor for HIV-1. Gb$_3$ is primarily expressed in renal endothelial and epithelial tissues, however peripheral blood mononuclear cells (PBMCs) were found to have enhanced Gb$_3$ expression after PHA/IL-2 activation. Activated PBMCs are predominantly CD4 positive T cells which are the primary targets for HIV-1 infection. Gb$_3$ is the sole receptor for verotoxin produced by Enterohemorrhagic E coli (EHEC). VT is a ribosomal inactivating protein (RIP) that has an AB$_5$ subunit structure. The single catalytic A subunit has a N-glycosidase activity that inhibits protein synthesis by ribosomal inactivation and results in cell death. The pentameric B subunits bind to receptor Gb$_3$ for internalization and subsequent retrograde transportation in susceptible cell types. Pilot experiments suggested treatment of PBMCs with VT1 during PHA/IL-2 activation results in significantly reduced viral production after subsequent HIV-1$_{IIIb}$ exposure without affecting overall cell viability. We hypothesized that VT can be a specific inhibitor for HIV-1 infection and can potentially be an effective treatment for HIV-1.

In testing the hypothesis, the inhibitory effect of VT 1 and 2 on HIV-1 productive infection was confirmed in both T-cell leukemia cell line JKT-C and primary PHA/IL-2 activated PBMCs. The anti-HIV-1 effect of VT was found to be receptor Gb$_3$ independent as verified by a Gb$_3$ negative cell line, Jurkat-C. The VT1A subunit alone was also found to be sufficient to confer viral resistance. In addition, experiments with PBMCs clearly showed that the VT1A subunit by itself was more potent than the holotoxin. Gb$_3$ independency was further verified by VT1B neutralizing anti-serum protection assays and that PBMCs were slightly more sensitive to VT1A treatment than the holotoxin. Contrary to previous findings, VT treatments of PBMCs reduced their cell proliferation rate in a dose dependent manner, and nascent protein synthesis was also reduced. Gene expression array analysis revealed that VT1A significantly altered the expression level of 44 genes in PBMCs, with adjusted P value of less than 0.03. Eight of them belong to
genes encoding histone cluster protein. Western blotting showed that global phospho-Tyr levels were unaffected.

In a separate study, the relationship between Gb3 and HIV-1 protection by VT was examined in Gb3 positive monocytic THP-1 cell line. THP-1 cells are very sensitive to the cytotoxicity effect of VT with an LC50 of 1pg/mL. Our lab recently found evidence suggesting Gb3 as a natural resistance factor for HIV-1 infection (Lund et al., 2009; Ramkumar et al., 2009). However, VT treatment also inferred resistance to HIV-1, which would have killed any Gb3 expressing cells. Thus a Gb3 low expression population of THP-1 cells was selected by culturing with VT. The resulting cells (selTHP-1) were almost completely resistant to HIV-1IIIB infection despite having a 60% reduction in cell surface Gb3 expression. It is possible that the residual Gb3 represents a distinct pool which is unavailable to ligand binding. FACS analysis has revealed that the HIV-1 co-receptor CXCR4 surface expression was also reduced in selTHP-1 cells. This finding is similar to the observation from PBMCs from Fabry patients, where cell surface CCR5 expression was reduced possibly as a consequence of impaired trafficking imposed by Gb3 internal accumulation.

5.2 Verotoxin as HIV-1 inhibitor and proposed mechanism of action

In this thesis, we have demonstrated that VT can be a potent inhibitor for productive X4 HIV-1 infection. Indeed 1 hour of pre-exposure to VT1 or the catalytic A subunit alone has reduced de novo X4 HIV-1 productive infection by at least 50% in both primary human PBMCs and Jurkat-C T cell line (Figure 3.5 and Figure 3.9). It was also concluded that the inhibition effect of VT is independent of its receptor Gb3, which suggests the potential benefit of VT treatment is not limited to Gb3 expressing cells. This is the first study to characterize the protective effect of VT against HIV-1 infection, which may offer a new approach in the treatment of the disease.

The exact mechanism on how VT is actually protecting cells against HIV-1 productive infection is not clear. Since VT was only present for a short time before the actual infection took place, it is suspected that the effect VT exerted on the cells is long lasting, even after the toxin is removed. Even though no detectable VT binding to cells were observed, the possibility that a
few molecules of VT may have remained or entered the cells cannot be excluded. This could significantly affect the experimental outcome, since only one molecule of VT is sufficient to exert its cytotoxic effect in susceptible cells (Tam & Lingwood, 2007). Since 50% reduction in cell proliferation and protein synthesis inhibition were observed in PBMCs after VT treatment, the toxin could have entered the cytosol by an unknown means, and acted through its classical ribosomal inactivating activity. T cell proliferation is a sign of activation, which is required for HIV-1 viral propagation along with active gene transcription (Brady et al., 2009; Rosenberg & Fauci, 1991). It is also possible that ribosomal inactivation rendered the cells incapable of synthesizing viral components.

In addition, it was not determined which step of HIV-1 infection/life cycle that VT was interfering. Results from microarray study suggest VT may be altering gene expression and signal pathways in PBMCs that could increase resistance to infection. The possible increase in chromatin histone content could induce a more closed chromatin structure thus inhibiting HIV-1 provirus integration and/or lead to dormancy. Also, up-regulation of pro-inflammatory cytokines has been observed after VT exposure. This could aid in the rapid clearing of HIV-1 infected cells by CD8+ T cell cytoxicity or activation associated apoptosis, thus preventing further transmission. However, additional experiments are required for confirmation.

5.3 Possible route of entry of verotoxin in receptor Gb3 negative cells

Since Jurkat-C T cells and 98% of the PHA/IL-2 activated PBMCs do not express Gb3, and VT B subunit was not required for its anti-HIV-1 effect, it is possible that an alternative entry route was involved other than the classical receptor mediated endocytosis and retrograde transport. One possible route of entry is through random fluid phase macropinocytosis. Macropinocytosis is a form of endocytosis where cells non-selectively ingest small volumes of extracellular fluid along with any dissolved molecules present. Macropinocytosis in T lymphoblasts has been quantitatively measured by Goldmacher et al (Goldmacher et al., 1986). It was found that the peak rate of macropinocytosis is reached just 10min after incubation at 37°C. Approximately 3 to 4 fL of fluid is taken in during that time, which converts to approximately 26 to 35 molecules of VT holotoxin according to the concentration of VT used for our experiments. This is more than
sufficient to elicit major effects within the T cells; since only one molecule of VT is required to induce cytotoxicity within susceptible cell types (Tam & Lingwood, 2007). Although the size of VT holotoxin (MW: 68kDa) is larger than the commonly used pinocytotic uptake marker horseradish peroxidase (MW: 44kDa), the catalytic A subunit (MW: 32kDa) itself is much smaller. VT A could easily be taken up by macropinocytosis, which may be a reason for its enhanced potency compared to the holotoxin. In addition, it is possible that PHA/IL2 activated PBMCs have much more robust rate of macropinocytosis than a lab maintained cell line such as Jurkat-C, since VT only affected the proliferation and protein synthesis of PBMCs but not Jurkat-C.

5.4 Role of Gb3 in HIV-1 infection

Conflicting theories exist for the role of Gb3 in HIV-1 infection. Studies performed by the group of Puri et al and Fantini et al suggest Gb3 is an accessory receptor for HIV-1. On the contrary, previous findings in our lab using genetic and pharmacology manipulation studies have given rise to the theory that increased Gb3 expression correlates with enhanced resistance to HIV-1 infection. To further examine our theory in this thesis, a subpopulation of THP-1 cells that neutrally has little Gb3 expression was selected using VT. However, infection results with the selected cells found them to be completely resistant to HIV-lIIIb infection. One possible explanation was that by selecting VT resistance, we have also selected for cells with low lipid raft content. Because VT exerts its cytotoxicity through receptor mediated endocytosis within lipid rafts, cells with abundant receptor Gb3 in raft structure are eliminated in the VT selection, leaving behind cells where Gb3 is localized in non-raft regions or these cells have very little raft structure. Furthermore, since lipid raft is involved in many stages of HIV-1 infection, including initial virus to host cell fusion and eventual budding of progeny virus, lost of raft structure renders the cells resistant to productive infection.

Another interesting observation was that VT selected THP-1 cells have reduced cell surface CXCR4 expression which could further inhibit HIV-1 infection by reduced co-receptor availability. This could be a side effect of disrupted raft structure. This finding is similar to the observed reduction in cell surface CCR5 expression in Fabry disease PBMCs which could be a result of Gb3 accumulation (Lund et al., 2005). In the literature, GSL biosynthesis and recycling
are linked with sorting and trafficking of membrane associated proteins. The formation of lipid rafts and posttranslational modification of membrane proteins both occur within the Golgi network, which allows proteins destined for plasma membrane to be delivered in raft enriched vesicles (Simons & Ikonen, 1997; Smart et al., 1999).

The outer leaflet of plasma membrane is enriched in GSL in raft like microdomains. These microdomains are also found to be important in intracellular sorting of endocytic cargos (Simons & van Meer, 1988). Changes in GSL expression lead to profound changes in protein trafficking, where most observations come from studies of lipid storage diseases. For instance, Niemann-Pick type C (NPC) and Gaucher disease cells have altered endocytic transport that mistargets vesicles to lysosomes instead of the Golgi (Sillence et al., 2002; te Vruchte et al., 2004). A broad screening procedure for lipid storage diseases has been proposed which uses fluorescence labeled LacCer to detect mislocation of endocytic vesicles (Chen et al., 1999). Endocytic sorting is also vital for normal cell physiology because many cell surface markers and receptors rely on this process for cycling back to the membrane after ligand induced endocytosis. Therefore, special considerations are need when manipulating GSL expression and caution should be taken for interpreting results of such study.

5.5 Using verotoxin as treatment for AIDS

The current standard treatment for AIDS is HARRT, which has significantly improved the life quality and expectancy for patients. However, due to the expansive nature of this treatment, accessibility is still an issue in the poverty stricken Sub-Saharan countries, where it is most severely affected by AIDS. In addition, despite the continual effort at developing new ARV drugs, drug resistance remains a challenge for eradicating the disease as a result of the extremely rapid mutation rate of the HIV-1 virus. Therefore, it is necessary to search for new and more affordable approaches for treating AIDS. One alternative approach that has come to the attention of researchers is exploiting the anti-viral property of a group of plant or bacterial toxins, known as the ribosomal inactivating proteins (RIP). The anti-HIV-1 effect of RIs including: ricin, Poke-weed antiviral protein, Pseudomonas exotoxin-A and trichosanthin has been studied with some in vitro success (Chaudhary et al., 1988; McGrath et al., 1989; Olson et al., 1991; Till et
al., 1989). Because these toxins are eliminated by neutralizing antibodies within the body, they are proposed to be used in combination for prolonged treatment of HIV-1.

In this thesis, we have identified verotoxin as a potential candidate for an alternative HIV-1 treatment program involving RIPs. However, it is still a long way before VT could actually be used in patients. The major concern is the potential cytotoxic effect that VT has in human body, since its source, EHEC, is the cause for hemorrhagic colitis and hemolytic uremic syndrome. It is important to note that severe disease is usually associated with young children and the elderly; most people recover completely. Also, the primary disease target of EHEC is Gb3 positive renal epithelial cells. However this is receptor dependent and the A subunit alone is considered completely nontoxic. In our study, the catalytic A subunit was shown to be more potent than the holotoxin, this would eliminate potential side effects associated with receptor Gb3 positive host cell targeting by VT B subunit. It will also be beneficial to determine whether the N-glycosidase activity of the A subunit is required for the HIV-1 inhibition effect. Using catalytically inactive VT could further reduce the risk of unwanted side effects. This treatment could be used as a salvage program when the patient failed to respond to other ARV drugs. In addition, VT may offer a new approach to further our understanding of HIV-1 pathogenesis and develop new target for treatment. VT treatment on human PBMCs altered the expression level of 49 genes/probes as revealed by microarray analysis. Follow up experiments will aim to determine candidate genes that are linked to HIV-1 infection. One approach is to identify genes affected by both VT and viral exposure. They could be new targets for developing drugs that elicit the same desired effect with improved delivery and selectivity.

5.6 Future directions

In this thesis we have identified VT as a potent inhibitor for de novo X4 tropic HIV-1 productive infection in primary human PBMCs and Jurkat-C T cell line. However, many questions remain unanswered regarding the mechanism of inhibition. Here are a few experiments that could help address them:

1. Microarray results for VT treatment of PBMCs suggest potential up-regulation of histone cluster proteins and pro-inflammation cytokines, which need to be confirmed by real-time
PCR. Confirmed genes will then be cross referenced with list of genes affected by viral exposure, to determine possible gene targets for suppressing HIV-1 infection.

2. To verify that up-regulation of histone is linked to HIV-1 resistance, the HDAC inhibitor SAHA was used to treat PBMCs along with VT incubation. Since SAHA treatment inhibits cell proliferation, a safe dosage need to be determined. Other HDAC inhibitor could also be tested to find a suitable treatment.

3. The route of entry for VT into Gb3 negative cells are proposed to be random fluid phase macropinocytosis. This theory can be tested by monitoring changes in protein synthesis after treating the PBMCs with ethylisopropylamiloride. Macropinocytosis is dependent on the activation of p21-activated kinase 1 (Pak1) (Dharmawardhane et al., 2000), which is activated by small GTPase Rac (Knaus et al., 1998). Sodium/proton exchanger inhibitors, such as ethylisopropylamiloride, blocks Rac activation by decreasing cytosolic pH (Koivusalo et al., 2010; West et al., 1989).

4. The inhibitory effect of VT was only tested for X4 HIV-1IIIb virus. It is necessary to expand the infection experiment to R5 tropic viruses. This will determine whether VT can be used for preventive treatments since R5 viruses are responsible for the initial transmission of the disease.

5. Treatment of VT A subunit reduced cell proliferation and protein synthesis of PBMCs. Thus, it is beneficial to determining whether the enzymatic activity of A subunit is required for its anti-HIV-1 effect. This could be tested using catalytically inactive mutant VT1A or pre-incubation with neutralizing peptides that binds within the catalytic site.

6. The total HIV-1 resistance of selTHP-1 cells could be caused by disruption of lipid rafts or by non-raft localization of Gb3. The lipid raft content of selTHP-1 cells can be compared to wild type THP-1 cells by raft marker GM1 labeling. Percentage of Gb3 localized to raft fraction can be determined by comparing GSL extraction from Triton X-100 resistant fraction versus total cell extract extraction using VT-overlay. To determine if Gb3 reduction is the actual cause of HIV-1 resistance, infection should be performed after Gb3 is reintroduced into the plasma membrane of selTHP-1 cells.
6 References


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## Appendices

Table 1: Differentially expressed genes of VT1A treated PBMCs vs. control PBMCs.

Genes/probes with adjusted p value of less than 0.1 are listed. Fold change represents expression level difference between VT1A treated samples vs. control no VT samples. Positive values indicate an increase and negative values indicate a decrease.

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<th>Definition</th>
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<th>adj. p value</th>
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