CHARACTERIZATION OF HIV-1 BINDING TO PERIPHERAL BLOOD MONONUCLEAR CELLS VERSUS MONOCYTES/MACROPHAGES: RELATIONSHIP TO NEUROPATHOGENESIS

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DEDICATION

This Master of Science Thesis is dedicated to my lovely daughter, Lillian Leilani Munsaka, whose smile gave me the driving force to conduct research and write up this manuscript. I also dedicate this Thesis to my loving, caring and understanding wife, Fungai Harriet Lungu, who allowed me to work odd hours in the laboratory and did most of the house work as I toiled to get work done for this write up. Finally to my parents, Mr. Solomon and Mrs. Esther Munsaka, who gave me life and the best gift one can give a child; education, I say ndalumba kapati.
ACKNOWLEDGEMENTS

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ABSTRACT

Individuals with HIV-1-associated dementia (HAD) are characterized with increased percentages of circulating activated monocytes/macrophages (M/MΦ) with CD14/CD16 phenotype. Higher levels of HIV-1 DNA are detected in these activated cells, thus hypothesizing that the activated M/MΦ have higher viral binding and possibly leading to more permissive infectivity.

From a non-HIV-1-infected volunteer, peripheral blood mononuclear cells (PBMCs), magnetic bead-separated activated and non-activated monocytes were exposed to 2ng p24 units of LAI (X4 Strain) and p89.6 (dual tropic but preferentially X5 strain) for one hour at 37°C, 5% CO2. Viral binding capacity was assayed by RT-PCR using HIV Gag and β-actin primers with appropriate positive and negative control RNA and densitometry. Differences in binding capacities between each of the two groups were considered significant by Student’s t-test and One-Way ANOVA if p<0.05.

As expected, M/MΦ displayed a higher HIV-1 binding to p89.6 than to LAI, 0.497 vs. 0.328 (p=0.025), respectively. In the PBMCs, viral binding capacity was increased compared to M/MΦ, for LAI: 0.492 vs. 0.328, respectively (p=0.011); for p89.6: 0.878 vs. 0.497, respectively (p=0.004). Of note was the significantly higher binding found with p89.6 (0.878) compared to LAI (0.492) (p=0.004), since the PBMCs were from the same volunteer obtained at the same time. There was a trend for HIV-1 binding to be higher for activated monocytes [LAI (0.324), P89.6 (0.277)] compared to non-activated monocytes [LAI (0.225), p89.6 (0.249)], p= 0.362.
These results demonstrate that peripheral M/MΦ preferentially bind CCR5 virus suggesting that the high HIV DNA found in PBMCs represents bound virus on the M/MΦ subset. The enhanced binding of CCR5 strains to M/MΦ, particularly to activated M/MΦ, may lead to more permissive infection of this subset. The theory that increased trafficking of HIV-1-infected activated M/MΦ to the central nervous system is consistent with the findings.
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<th>Long Description</th>
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<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µL</td>
<td>microliter</td>
</tr>
<tr>
<td>A</td>
<td>Absorbance</td>
</tr>
<tr>
<td>BFA</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>copy of deoxyribonucleic acid</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FACS</td>
<td>flow activated cell sorting</td>
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<tr>
<td>Fc</td>
<td>crystallizable fragment of an antibody</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>HAART</td>
<td>highly active antiretroviral therapy</td>
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<tr>
<td>HAD</td>
<td>HIV-1-associated dementia</td>
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<tr>
<td>HIV DNA</td>
<td>HIV-1 DNA copy number</td>
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<tr>
<td>HIV-1</td>
<td>human immunodeficiency virus, type 1</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>MIP-1</td>
<td>monocyte inflammatory protein 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>M-CSF</td>
<td>macrophage colony stimulatory factor</td>
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<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage-colony stimulatory factor</td>
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<tr>
<td>mL</td>
<td>milliliter</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<td>NK cell</td>
<td>natural killer cell</td>
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<tr>
<td>p</td>
<td>probability</td>
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<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>RNase</td>
<td>ribonuclease</td>
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<tr>
<td>rpm</td>
<td>revolutions/rotations per minute</td>
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<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
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<tr>
<td>TAE</td>
<td>tris-HCl-acetic acid-EDTA</td>
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<tr>
<td>TLR</td>
<td>toll-like receptor</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-alpha</td>
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CHAPTER 1. INTRODUCTION

1.1 Background

Human immunodeficiency virus-type-1 (HIV-1)-associated dementia (HAD) continues to be a clinically important issue in the era of highly active antiretroviral therapy (HAART). While HAART has been successful in increasing longevity and quality of life within the HIV/AIDS population, the prolonged length of life is accompanied by an increased likelihood of developing HAD [1]. Also, HAART does not prevent but reduces HIV-related neurodegeneration [2]. This has changed the epidemiology of HIV in the developed countries where HAART is widely used. Greater numbers of people are living and aging with HIV as a chronic illness [1, 3]. Prior to HAART, the prevalence of HAD was estimated to be between 20% and 30% in patients with advanced HIV-1 disease [4]. With the widespread use of HAART in developed countries, the incidence of reported cases of HAD has declined by up to 53% [5]. Paradoxically, while the incidence of HAD has declined, the prevalence of HAD has not changed but rather appears to be increasing as patients live longer [2, 6, 7]. The prevalence of HAD is approximately 30% in HIV-1 infected cases [7]. The spectrum of the disease has also changed with dementia occurring at higher CD4 counts, increased number of cases of milder degrees of cognitive impairment, and greater variation in disease course [7]. This warrants further research into the pathogenesis of HAD. The significance lies in the knowledge to be gained on how HIV-1 infection of activated monocytes may lead to HAD, why HAD continues to be seen in the HAART era and the potential that HIV-1 infection in activated monocytes may represent a new therapeutic target for prevention or treatment of HAD. It has been
hypothesized that activation of HIV-1-infected peripheral blood monocytes/macrophages with their subsequent migration to the central nervous system (CNS) plays a key role in the pathogenesis of HAD. Activated monocytes/macrophages produce proinflammatory cytokines that play an important role in neuropathogenesis [8, 9]. Inflammatory cytokines, chemokines and other mediators produced by HIV-1-infected activated monocytes may directly or indirectly contribute to neuronal damage or apoptosis [10].

The current research focused on HIV-1 infection of monocytes/macrophages and concentrated on the first step involved in infection, namely viral binding. The central hypothesis was that activated monocytes increased surface expression of HIV-1 co-receptors making them bind more virus and thus become more permissive to infection. HIV-1-infected activated monocytes/macrophages are known to traffic to the CNS [11, 12] where they play a key role in the neuropathogenesis. HIV-1 proviral DNA copy number in peripheral blood mononuclear cells (PBMCs) has been correlated with HAD [13] and thus HIV DNA may represent an entity central to the pathogenesis of HAD. Therefore, HIV DNA in activated monocytes/macrophages outside the CNS may play a key role in the neuropathogenesis of HIV-1.

1.2 HIV-1 Tropism

HIV infects cells of the lymphoid and myeloid lineage primarily by attaching its glycoprotein 120 (gp120) and glycoprotein 41 (gp41) fusion protein to the CD4 cell surface receptor and chemokine co-receptors CXCR4 and CCR5 respectively [14, 15]. CCR5 is a member of the β-chemokine receptor family, which is predicted to be a seven
transmembrane protein similar to G protein-coupled receptors. This protein is expressed by T cells and macrophages, and is an important co-receptor for macrophage-tropic HIV, to enter host cells [16]. Defective alleles of this gene have been associated with the HIV infection resistance [17, 18]. The ligands of this receptor include monocyte chemoattractant protein 2 (MCP-2), macrophage inflammatory protein 1 alpha (MIP-1 alpha), macrophage inflammatory protein 1 beta (MIP-1 beta) and regulated on activation normal T expressed and secreted protein (RANTES) [19]. These chemokines are involved in recruitment of lymphocytes during inflammation. CXCR4, also called fusin, is an alpha-chemokine receptor specific for stromal-derived-factor-1 (SDF-1 also called CXCL12), a molecule endowed with potent chemotactic activity for lymphocytes. This receptor is one of several chemokine receptors that HIV isolates can use to infect CD4+ T cells. Traditionally, HIV isolates that use CXCR4 are known as T-cell tropic isolates. Typically these viruses are found late in infection.

HIV-1 infection is marked by depletion of CD4+ T-cells resulting in immunocompromise of infected individuals [20]. Proposed mechanisms for HIV killing of T cells include the formation of giant cell syncytia through the interactions of gp120 with CD4 and chemokine receptors [21], the accumulation of unintegrated linear forms of viral DNA, the proapoptotic effects of the Tat [22], Nef, [23], and Vpr proteins [24], and the adverse effects conferred by the metabolic burden that HIV replication places on the infected cell [25].
1.3 Neuropathogenesis of HIV-1

HIV-1-associated dementia is one of the most common and clinically important CNS complications of late HIV-1 infection [3]. It is a source of great morbidity and is an important risk factor for mortality. While its pathogenesis remains unclear, HAD is generally thought to be caused by HIV-1 itself, rather than a result of opportunistic infections [26-28]. It has been shown that individuals with HAD have higher percentages of circulating activated monocytes/macrophages (CD14+/CD16+) [8, 26-28]. Circulating proviral HIV DNA in PBMCs has also been associated with HAD [13]. It has been shown recently that activated monocytes (CD14+/CD16+) are more permissive to infection and preferentially harbor HIV-1 in vivo and in vitro [29]. Activated monocytes have a proinflammatory cytokine profile and may have increased CD16 expression.

Monocytes/macrophages become infected with HIV-1 via CD4 cell surface receptor and CCR5 chemokine co-receptor [15, 17, 30]. Increasing evidence in the literature supports the theory that monocytes/macrophages harbor the virus and serve as potential reservoirs of infection [29, 31-33]. Monocytes/macrophages are also believed to play a role in the pathogenesis of HAD as they are implicated to carry HIV-1 into the central nervous system in a ‘Trojan horse’ mechanism [10].
It is not clear how activated monocytes/macrophages induce neuronal injury. Both direct and indirect neurotoxic mechanisms are believed to occur [34]. Direct neuronal toxic mechanisms involve release of viral molecules (gp120, Tat, Vpr) shed by HIV from infected monocytes/macrophages and microglia that directly injure neurons. Indirect mechanisms involve influx of activated monocytes/macrophages that activate perivascular microglia to release proinflammatory cytokines and chemokines (interleukin-1β and tumor necrosis-α) and other excitatory mediators (TRAIL, glutamate, quinolinic acid) that induce neuronal apoptosis [2, 34-36]. Proinflammatory cytokines increase expression of adhesion factors on brain endothelial cells enabling further transmigration of infected and activated monocytes/macrophages into the brain [10, 37]. Furthermore, activation and excitation of astrocytes modifies the permeability of the blood-brain barrier and promotes the migration of more monocytes into the brain [10].

Although the severity and relative prominence of some symptoms and signs may vary among individual patients, the general character of HAD involves three functional categories: cognition, motor performance, and behavior [38]. Cognitive symptoms include low attention, diminished concentration, impaired short-term memory, impaired information processing and language difficulties. Motor symptoms include slow movements, abnormal gait and hypertonia. Behavior symptoms include irritability, emotional lability, personality changes, intellectual apathy and social withdrawal.
1.4 The Role of Monocytes/Macrophages in Neuropathogenesis of HIV-1

Circulating monocytes originate from the bone marrow and circulate in the blood for about three to five days before migrating and differentiating into tissue macrophages [39-41]. Monocytes in the brain differentiate into microglia [42]. Microglia are thought to be a source of HIV-1 to cells in the brain like astrocytes and oligodendrocytes. HIV-1 establishes a persistent infection in astrocytes rather than a productive one leading to astrocytes activation [43]. Astrocytes upon activation produce proinflammatory cytokines and disrupt the integrity of the blood-brain-barrier enabling further migration of activated monocytes/macrophages into the CNS. Sub-cortical astrocytes may therefore be an unrecognized reservoir for HIV-1 and their inflammatory process may play a role in the neuropathogenesis. Monocytes typically express CD14, a lipopolysaccharide receptor and CD16, Fc gamma III receptor in low amounts. Upon activation or during inflammation CD16 expression is increased in monocytes [44, 45]. The percentage of CD16+ monocytes increases with HIV-1 infection and it has been suggested that these monocytes play a role in disease progression via cytokine production and other cell-to-cell interactions with other immune cells [46]. HIV-1 is not cytotoxic to monocytes and thus allows them to continue to harbor HIV-1 [33, 47]. HIV-1 persists in monocytes even though infected individuals are effectively treated with HAART [45].
HIV-1-infection has also been shown to increase expression of chemoattractant cytokines (chemokines). Two chemokine beta peptides, macrophage inflammatory protein 1 alpha and 1 beta (MIP-1-α and MIP-1-β) are induced in monocytes following HIV-1 infection [48]. Monocyte activation has also been reported to increase surface expression of CCR5 [49-51]. CCR5 is an important co-receptor for macrophage-tropic HIV. Increased surface expression of chemokines on HIV-1-infected activated monocytes makes them traffic more into the central nervous system [11, 12]. The CD16+ phenotype may also increase HIV-1 infectivity of monocytes/macrophages [29]. The increased HIV-1 infectivity of activated monocytes/macrophages may be due to increased HIV-1 binding efficiency of activated monocytes/macrophages compared to non-activated monocytes/macrophages. The enhanced HIV-1 infectivity of activated monocytes makes them harbor more proviral HIV DNA. Circulating proviral HIV DNA has been associated to HAD [13]. HIV-1-infected monocytes/macrophages have been thought to have increased trafficking into the central nervous system where they play a key role in neuropathogenesis. The master's thesis aims to fill in the knowledge to what makes monocytes harbor more proviral DNA. The activated monocyte fraction is suggested to be the primary source of HIV proviral DNA. The overall goal of this study is to establish the mechanistic link between HIV-1 infection of non-activated monocytes/macrophages (CD14+/CD16⁻), activated monocytes/macrophages (CD14+/CD16⁺) and HIV-1-associated dementia.
1.5 Thesis Objectives

The long term goal of this study is to establish the mechanistic link between HIV-1 infection of non-activated monocytes/macrophages (CD14+/CD16−), activated monocytes/macrophages (CD14+/CD16+) and HIV-1-associated dementia.

Specific Aims for the Master Thesis include:

Specific Aim #1: To evaluate HIV-1 binding to PBMCs compared to monocytes using LAI and p89.6 HIV-1 strains.

Hypothesis: HIV-1 LAI will bind more to PBMCs than monocytes compared to p89.6. HIV-1 p89.6 will bind more to monocytes than PBMCs compared with LAI.

Rationale: LAI is a T-lymphocyte-tropic virus and so it will bind preferentially to PBMCs [52]. PBMCs largely comprise lymphocytes. p89.6 is a dual-tropic virus but preferentially infects monocytes/macrophages and therefore will have a higher viral binding capacity to monocytes [53].
Specific Aim # 2: To evaluate CD16 expression on monocytes/macrophages grown in culture over a seven day period without stimulation and to characterize baseline CD16 expression on cultured monocytes. To develop a non-activating culture system for monocytes

Hypothesis: There will be an increase in levels of CD16 expression on monocytes grown in culture without any stimulation.

Rationale: Monocytes/macrophages require stimulation to increase surface expression of activation markers like CD16. Culture components such as media constituents and cultureware are known to activate monocytes [54]. It is important to know if there is change in expression of CD16 in monocytes grown in culture overtime as viral binding assays involved characterization of HIV-1 binding to CD16⁺ versus CD16⁻ monocytes.
Specific Aim # 3: To activate monocytes in vitro using lipopolysaccharide (LPS). To develop an artificial system for activating monocytes

Hypothesis: Lipopolysaccharide stimulation of monocytes will increase surface expression of CCR5 and activation markers such as CD16.

Rationale: Lipopolysaccharide (LPS, also called endotoxin), a component of the outer membrane of gram-negative bacteria, is the most potent and well-characterized gram-negative bacterial signal molecule. LPS interacts with CD14 together with Toll-like receptor 4 (Tlr4) molecule on monocytes/macrophages and activates secondary messenger signaling transduction pathways [38-41] that result in activation. Activation of monocytes has been shown to increase surface expression of CCR5. CCR5 is an important co-receptor for macrophage-tropic HIV.
Specific Aim # 4: To characterize HIV-1 binding to activated monocytes vs non-activated monocytes using LAI and p89.6 viral strains.

Hypotheses: HIV-1 binding to activated monocytes (CD14+/CD16+) will be higher than to non-activated monocytes (CD14+/CD16-).

Rationale: There is increased surface expression of CD16 and CCR5 on monocytes during activation [16, 55]. This may enhance binding of HIV-1 strains that preferentially use CCR5 HIV co-receptor.
CHAPTER 2. METHODS

Specific Aim #1: To evaluate HIV-1 binding to PBMCs compared to monocytes using LAI and p89.6 HIV-1 strains.

Hypothesis: HIV-1 LAI will bind more to PBMCs than monocytes compared to p89.6.
HIV-1 p89.6 will bind more to monocytes than PBMCs compared with LAI.

Rationale: LAI is a CNS derived T-lymphocyte-tropic virus and so it will bind preferentially to PBMCs. PBMCs largely comprise lymphocytes. p89.6 is a CNS derived dual-tropic virus but preferentially infects monocytes/macrophages and therefore will have a higher viral binding capacity to monocytes.

2.1 Experimental Design for Specific Aim #1

Monocytes/macrophages were isolated and purified from fresh whole blood from HIV-1-negative subjects using Ficoll, Percoll and Dynal magnetic beads selection. Monocytes isolated by Dynal magnetic beads selection and PBMCs from Ficoll centrifugation were exposed to 2ng p24 units of LAI and p89.6 HIV-1 strains for 1 hour in triplicate. Viral binding was assessed from the cells by RT-PCR using β-actin and HIV-1 gag primers.
2.2 Isolation of Peripheral Blood Mononuclear Cells (PBMCs) by Ficoll Centrifugation

Ten tubes of whole blood were obtained from healthy donors in 10mL heparin tubes following approval of the study by the University of Hawaii Institutional Review Board, CHS# 14313. The blood was transferred into 50mL conical tubes using a serological pipette. The blood was then spun in a centrifuge at 1200rpm for 10 minutes at room temperature. The upper plasma layer was removed with a Pasteur pipette up to 1cm from the red blood cell layer. The red blood cell fraction was diluted 1:1 with Dubeco’s phosphate buffered saline (D-PBS) and then 35mL of the mixture was layered gently onto 15ml Ficoll-paque (Amersham Biosciences Inc, Piscataway, NJ) in a 50mL conical tube [56]. The tubes were spun at 2000rpm with brake off for 25 minutes at 4°C. The PBMCs in the interface layer (buffy coat) were recovered using a Pasteur pipette and washed twice with up to 50mL 2% fetal bovine serum (FBS) and PBS to remove platelets. The cells were spun at 1200rpm for 10 minutes at 4°C for washing. The supernatant was decanted and discarded and then the tubes were agitated to break the cell pellet. The cells were re-suspended in 20mL of 2% FBS/PBS and a 50μL aliquot was stained with trypan blue for counting using a hemocytometer.

An aliquot of 5x10^5 cells was stained with 20μL anti-CD14-FITC and 20μL anti-CD16-PE for 20 minutes, washed twice and fixed in 1% Para-formaldehyde. The Cells were analyzed using FACSCaliber (BD Biosciences, San Jose, CA) or FACSaria (BD Biosciences, San Jose, CA) flow cytometry to determine purity.
2.3 Isolation of monocytes from PBMCs using single gradient Ficoll followed by double gradient Percoll gradient centrifugation and Dynal Magnetic Beads

PBMCs obtained from the Ficoll procedure were suspended in 4mL 2% FBS, 1x Hanks solution (HBSS with 2% FBS). The cells were then layered on a double gradient Percoll (GE Healthcare Bio-sciences Corp., Piscataway, NJ) comprising 5mL 37% Percoll (top) and 5ml 50% Percoll (bottom) in a 15mL conical tube. The cells were spun at 2000rpm with brake off for 25 minutes at 4°C. The monocytes in the interface layer were recovered carefully with a transfer pipette and placed in 15mL conical. 2% FBS/1X PBS was added up to 10mL to wash. The mixture was spun at 1200rpm for 10 minutes at 4°C. The supernatant was discarded and then the tube was agitated to break the cell pellet. The cells were treated with Accumax (Innovative Cell Technologies Inc, San Diego, CA) 1:1 to prevent clumping of cells and washed with 2% FBS/1x PBS.

An aliquot of 5x10⁵ cells was stained with 20μL anti-CD14-FITC and 20μL anti-CD16-PE for 20 minutes, washed twice and fixed in 1% Para-formaldehyde. The cells were analyzed using FACSCaliber or FACS_Aria flow cytometry to determine purity.

200μL of fresh monocytes obtained from Percoll gradients were suspended in 1mL 2%FBS, 1XPBS and 20μL per 10x10⁶ cells of the blocking reagent containing gamma globulin was added to prevent non-specific binding by blocking monocyte Fc-receptors for 5 minutes. A cocktail antibody mix (containing mouse monoclonal antibodies for CD2, CD7, CD16a, CD16b, CD19, CD56 and CD235a) was added to deplete T cells, B cells, NK cells, erythrocytes and granulocytes. The mixture was incubated at 4°C on a
rotary shaker for 20 minutes. The cells were washed twice with up to 4mL 2%FBS/PBS, centrifuged at 1200rpm for 10 minutes at 4°C and re-suspended in 2mL 2%FBS/PBS. Then 1mL of the pre-washed Dynal magnetic beads (Invitrogen Cooperation, Carlsbad, CA), uniform supermagnetic polystyrene beads coated with Fc-specific receptor against mouse IgG, were added to the cells with thorough mixing. The cells were incubated for 20 minutes with gentle tilting and rotation at 4°C. After incubation, the tubes were placed on a magnet (Dynal MPC) for two minutes and then the supernatant (negatively isolated monocytes) was pipetted out using a Pasteur pipette into a new tube. Recovered monocytes were washed with 2%FBS/PBS. Aliquots were stained for flow cytometry and counting while the rest of the cells were cultured for viral binding and other downstream experiments.

2.4 Viral Binding Assay

2.4.1 Viral Titration

1.0 x10^6 PBMCs and monocytes in duplicate were suspended in 185μL, 180μL, 175μL, 150μL, 100μL and 200μL of HL-1 complete media supplemented with 10% FBS, 100 units/mL penicillin/Streptomycin, 4mM L-glutamine and 5mM HEPES and added to wells in a 24 well plate. HIV-1 viral stock p89.6 (82.143ng/mL P24 units) was quick thawed in a water bath at 37°C added to the cells in the following amounts; 1ng, 2ng, 3ng, 4ng p24 equivalent units and no virus in the negative controls. The cells were thoroughly mixed with a Pasteur pipette and then incubated at 37°C, 5% CO₂ in the incubator for one hour. The cells were washed by adding PBS up to 1mL and then spun
in the centrifuge at 1200rpm for 10 minutes. 900μL of the media was sucked out gently using a pipette 900μl fresh PBS was added to repeat the wash. The washing was repeated three times. The PBS/media was completely removed during the last wash and then 350μL of lysis buffer was added to lyse the cells for RNA extraction. Saturation was evaluated by RT-PCR and densitometry.

2.4.2 Determination of washes to remove unbound virus

1.0 x10^6 PBMCs and monocytes in duplicates were suspended in 175μl, 150μl and 200μl HL-1 complete media and added to wells in a 24 well plate. HIV-1 viral stock p89.6 (82.143ng/μl P24 units) was quick thawed in a water bath at 37°C added to the cells in the following amounts; 2ng, 3ng and 4ng p24 units and no virus in the negative controls. The cells were thoroughly mixed with a Pasteur pipette and then incubated at 37°C, 5% CO₂ in the incubator for one hour. The cells were washed by adding PBS up to 1mL and then spun in the centrifuge at 1200rpm for 10 minutes. 900μl of the media was sucked out gently using a pipette and 900μl of fresh PBS was added to repeat the wash. The supernatants were saved for RNA extraction. The washing was repeated five times and five supernatants were saved for RNA isolation and RT-PCR. The PBS/media was completely removed during the last wash and then 350μL of lysis buffer was added to lyse the cells for RNA extraction. HIV presence in the supernatants was assayed by RT-PCR.
2.4.3 Evaluation of HIV-1 binding to PBMCs compared to monocytes using LAI and p89.6 HIV-1 strains.

Fresh PBMCs and monocytes obtained from Percoll and Dynal magnetic beads separation were suspended 200μL RPMI supplemented with 10%FBS, 5ng/mL M-CSF, 100 units/mL penicillin/Streptomycin, 4mM L-glutamine and 5mM HEPES buffer. X-Vivo media supplemented with 10% normal human serum was also used to culture monocytes. The cells were cultured in a 96 well polypropylene plate at 5 x 10⁵ cells/well and immediately HIV-1 was added. The viral binding assay was performed as described [57]. Negative controls were also set up without virus exposure. The cells were incubated for 1 hour to allow for viral binding. HIV-1 viral stock p89.6 (82.143ng/mL p24 units) and LAI (1,363.6ng/mL) (NIH AIDS Reagent Program, Germantown, MD) were used for viral binding assays. Viral concentration of 2ng p24 units was added in triplicate. The cells were thoroughly mixed with a transfer pipette and then incubated at 37°C, 5% CO₂ in the incubator for one hour. The plate was spun down at 1200rpm for 10 minutes at room temperature and 100μL of supernatant was removed using a Pasteur pipette. 200μL PBS was added to wash. The plate was spun down again to wash and the whole wash procedure was repeated three times. After the last wash, PBS was completely removed and 350uL lysis buffer was added to lyse the cells. The cell lysates were transferred in to 1.5 mL RNase free tubes. The wells were again rinsed with 350uL lysis buffer which was pooled in respective tubes. The lysis was repeated and the lysates were pooled to respective tubes. The lysates were either frozen down at -80°C or RNA was immediately extracted.
RNA was isolated using the RNeasy microkit (Qiagen Inc, Valencia, CA) according to the manufacture's specifications. The lysed cells were homogenized using a homogenizer for 30 seconds or passed through a Qiagen shredding column and spun in the micro-centrifuge at 10,000rpm for 2 minutes. 350µL of 70% ethanol was added and thorough mixing was done using a pipette to the shredded lysate. The mixture was applied to the RNeasy microkit spin column and spun in the micro-centrifuge at 10,000 rpm for 15 seconds. The flow through was discarded. The remaining shredded lysate was added to the column and centrifugation was repeated. The column was washed with 500µL buffer RW1 and treated with DNase 1 (Qiagen Inc, Valencia, CA) (10µL DNase-1 in 70µL buffer RDD) for 15 minutes at room temperature. Then 500µL of buffer RW1 was added onto the column and the tube was spun at 10,000rpm for 15 seconds to wash. The flow through was discarded. 500µL buffer RPE (with 70% ethanol added) was added to wash the RNA on the column. The tube was spun at 10,000rpm for 2 minutes. The spin column was further treated with 500µL 80% ethanol and spun at 10,000 rpm for 2 minutes. The flow through was discarded and then the column was placed in a new tube and spun at 10,000rpm for 5 minutes with caps open to dry the filter. The filter/spin column was transferred into a labeled collection tube and 14µL of RNase free water was added. The tube was left to react at room temperature for a minute and then spun in the micro-centrifuge at 10,000rpm for 1 minute to elute the RNA. The RNA was collected and stored in the -80°C freezer.
RNA purity and concentration was measured by using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). The A_{260/280} ratio was used to assess RNA quality. Good quality RNA of A_{260/280} ratio between 1.7 and 2.2 was used for RT-PCR and/or stored at -80°C.

To make cDNA equal amounts of good quality RNA (50ng) were used. The RNA was diluted with RNase free water to a total volume of 12μL. cDNA was made using a mixture Oligo dT and random hexamer primers and reverse transcriptase in the Roche Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). The reaction was run in the thermocycler at 55°C for 30 minutes and then 85°C to deactivate the reverse transcriptase and then held at 4°C. cDNA was either purified as below or was assessed for genomic DNA contamination and normalized by β-actin PCR and densitometry.

20μL cDNA reaction was added to 100μL binding buffer PB for purification. The mixture was mixed thoroughly and then applied to the Qiagen Qiaquick spin column (Qiagen Inc, Valencia, CA) and spun at 10,000rpm for 1 minute. The flow through was discarded. 750μL Buffer PE (with 70% ethanol) was added to the column. The column was spun in the microfuge at 10,000rpm for 1 minute. The flow through was discarded. The column was transferred into a new collection tube and further spun at 10,000rpm for 1 minute to dry the filter and remove residual ethanol. The spin column was transferred to a labeled collection tube and then 30μL DNase free water was added. The tube was left to
react at room temperature for 1 minute. The tube was then spun at 10,000rpm for 1 minute to elute the cDNA. The cDNA was stored in the -20°C freezer.

β-actin and HIV gag PCR were performed to determine viral binding. β–actin PCR was set up using β–actin forward and β–actin reverse primers (Table 1), which are specific for cDNA amplification and do not amplify genomic DNA [37]. PBMC cDNA was used as positive control where as PBMC DNA and water were used as negative controls. The tubes were run in the thermocycler using the Eppendorf master mix (2.5x) (Eppendorf, Hamburg, Germany) at 94°C hold for 1 minute and 35 cycles of 94°C for 1 minute (melting), 58.5°C for 30 second (annealing) and 72°C for 30 seconds (extension). Final extension was done at 72°C and then the reaction was held at 4°C.

HIV gag PCR was performed using HIV gag primers (Table 1) [58]. ACH-2 (HIV-1 chronically infected T-cell line) (NIH AIDS Reagent Program, Germantown, MD) cDNA was used as a positive control and PBMC DNA from a normal healthy individual and water as negative control. The tubes were run in the thermocycler at 95°C hold for 1 minute and 30 one step cycles of 95°C for 1 minute (melting), 57°C for 40 seconds (annealing and extension). Final extension was done at 72°C for 3 minutes and then the reaction was held at 4°C.
β-actin and HIV gag PCR products were resolved on 2.5% agarose gels. 1.5g SeaKem agarose (Cambrex Corp, East Rutherford, NJ) was added to 60mL TAE buffer (40mM Tris acetate, 1mM EDTA). The mixture was heated in the microwave for 1 minute. The molten agarose was allowed to cool at room temperature and then 0.5μg/mL Ethidium bromide was added. The molten agarose was poured into the electrophoresis chamber and allowed to solidify. TAE buffer was poured into the chamber until the agarose was submerged.

15μL sample was mixed with 3μL 6x loading dye and added to each well. 10μL 100bp bench top ladder (Invitrogen Corporation, Carlsbad, California) was used in the first well. The reaction was run at 90V for 1 hour and 30 minutes. The gel pictures were then taken using an Olympus digital camera.

The gel pictures obtained were scanned using a densitometer (Lumi-imager, Roche Applied Biosciences, Pleasanton, CA) to compare the amounts of PCR product by measuring the amount of light intensity of the bands. Gels or gel pictures were exposed for 10 seconds. Digital pictures of gels were also imported into the Lumi-imager. Background correction was set from negative controls. The band intensities were expressed in Boehringer/Biological Light Units (BLU). The HIV gag/β-actin ratio was calculated to normalize and compare relative intensities of the bands.
Viral binding capacity was assayed by RT-PCR using HIV \textit{gag} and \textit{\beta}-actin primers with appropriate positive and negative control RNA. Amplified fragments were resolved on 2.5\% agarose gels and analyzed by densitometry. From the scanned gels, the ratio of HIV \textit{gag} light units/\textit{\beta}-actin light units was compared between the two cell populations and the virus strains. Differences in binding capacities between each of the two groups were considered significant by Student’s t-test if \( p<0.05 \). Significance between different viral strain treatments were analyzed by one-way ANOVA and were considered significant if \( p<0.05 \). The statistics were performed using SigmaStat 3.0 (SPSS Inc). Three replicates were set up and the average ratio of HIV \textit{gag}/\textit{\beta}-action per replicate was considered in running statistics.
Specific Aim #2: To evaluate CD16 expression on monocytes/macrophages grown in culture over a seven day period without stimulation and to characterize initial CD16 expression on cultured monocytes. To develop a non-activating culture system for monocytes.

Hypothesis: There will be an increase in levels of CD16 expression on monocytes grown in culture without any stimulation.

Rationale: Monocytes/Macrophages require stimulation to increase expression of activation markers like CD16. However, culture conditions such as media components and cultureware are known to activate monocytes [54]. It is important to know if there is change in expression of CD16 in monocytes grown in culture overtime as viral binding assays involved characterization of HIV-1 binding to CD16+ versus CD16- monocytes.

2.5 Experimental Design for Specific Aim #2

Non-activated monocytes/macrophages were isolated and purified from fresh whole blood from HIV-1-negative subjects using Ficoll, Percoll and Dynal magnetic beads negative selection; placed in culture for 7 days; and analyzed by flow cytometry.
2.5.1 CD16 Expression Assay

Non-activated monocytes (CD14⁺/CD16⁺) obtained from Dynal Magnetic Beads isolation were re-suspended in 400μL RPMI supplemented with 10%FBS, 5ng/mL M-CSF, 100 units/mL penicillin/Streptomycin, 4mM L-glutamine and 5mM HEPES. The cells were then placed in a 96 well plate at 5 x 10⁵ cells/well in 400μL media and cultured over a seven day period in an incubator at 37°C and 5% CO₂. Media was changed every two days by spinning down the plate and pipetting off half of the media and replacing it with fresh media. The cells were harvested on each day starting at day zero, treated with Accumax (Innovative Cell Technologies Inc, San Diego, CA) 1:1 to prevent clumping of cells and washed with PBS. The cells were then stained with 20μL anti-CD14-FITC and anti-CD16-PE and fixed in 1% Para-formaldehyde for flow cytometry using FACSARia (BD Biosciences, San Jose, CA). Differences in CD16 expression between each of the days compared to day 0 were considered significant by Student’s t-test if p<0.05.
**Specific Aim # 3:** To activate monocytes in vitro using Lipopolysaccharide (LPS)

To develop an artificial system for activating monocytes

**Hypothesis:** Lipopolysaccharide stimulation of monocytes will increase surface expression of CCR5 and activation markers such as CD16.

**Rationale:** Lipopolysaccharide (LPS, also called endotoxin), a component of the outer membrane of gram-negative bacteria, is the most potent and well-characterized gram-negative bacterial signal molecule. LPS interacts with CD14 together with Toll-like receptor 4 (Tlr4) molecule on monocytes/macrophages and activates secondary messenger signaling transduction pathways [38-41] that result in activation. Activation of monocytes has been shown to increase surface expression of CCR5. CCR5 is an important co-receptor for macrophage-tropic HIV.

2.6 **Experimental Design for Specific Aim # 3**

Activation of cells was accomplished by initially priming whole blood with IL-10 followed by stimulation with LPS. The cells were then analyzed by flow cytometry for activation markers.
2.6.1 Whole blood stimulation

50mL of whole blood were collected from a healthy donor in 14:1 Citrate/Phosphate/Dextrose (CPD) as described [59]. The blood was transferred in two 75mL Teflon flasks and primed with recombinant human IL-10 (10ng/mL) at 37°C, 5% CO₂ for 20 hours. Teflon flasks were used to prevent adherence of monocytes. At the end of the priming period whole blood was diluted 1:3 with RPMI 1640 medium and 35mL was layered on 15mL Ficoll in a 50mL conical tube. The tubes were centrifuged at 2000rpm for 25 minutes at 15°C. The PBMCs were recovered from the buffy coat using transfer pipette. The PBMCs were washed twice to remove platelets and re-suspended in RPMI supplemented with antibiotics (100IU/mL Penicillin, 100ng/mL Streptomycin), 0.2% heat-inactivated normal human serum and 1μg/mL Indomethacin (Sigma Aldrich, St. Louis, Missouri), to get 6 x 10⁶ cells/mL. Indomethacin was dissolved in 60% DMSO and 40% saline as described [60]. PBMCs were then incubated in Teflon flasks at 37°C, 5% CO₂ for 20 hours in the presence of 2μg/mL Escherichia coli LPS O111:B4 (Sigma Aldrich, St. Louis, Missouri). At the end of the culture period, monocytes were separated by Percoll centrifugation and enriched using Easysep human monocytes enrichment without CD16 depletion (Stem Cell Technologies, Vancouver, Canada).

Monocytes recovered from Percoll centrifugation were suspended in 100μL Robosep buffer (PBS, 2% FBS, 1mM EDTA). 10μL of αCD32 blocking antibody (Stem Cell Technologies, Vancouver, Canada) was added to prevent non-specific binding to monocytes by blocking monocyte Fc receptors. The tubes were incubated at 4°C for 5
minutes. 5μL of the antibody cocktail 'without CD16 depletion' was added the tubes were incubated at 4°C for 5 minutes. At the end of the incubation period 5μL of the magnetic particles were added to the cells then Robosep buffer was added up to 2.5mL. The cells were incubated at 4°C for 5 minutes and then the tubes were placed in a magnetic for 2.5 minutes. The supernatant (unbound monocytes) was poured off into a fresh FACS tube. The cells were re-suspended in 2.5mL Robosep buffer and the selection was repeated. The recovered monocytes were washed twice with 2%FBS/PBS. Aliquots were stained for flow cytometry and counting while the rest of the cells were cultured for viral binding and other downstream assays.

An aliquot of cells (5x10⁵) was washed and re-suspended in 100μL PBS, 2% FBS. Antibodies conjugated to fluorescent dyes (anti-CD14-FITC, anti-CD16-Alexa 674, anti-CCR5-APC Cys7, anti-CD4-PE Texas Red, and anti-CXCR4-PE) were added according to manufacturers’ specifications as well as their fluorescence spectrum. The cells were incubated for 20 minutes at room temperature away from light. The cells were washed twice with up to 3mL PBS, 2% FBS twice. The cells were then fixed in 1% Para formaldehyde, 2% FBS. To transfer the cells from the plate to FACS tubes, the cells were treated with Accutase (Innovative Cell Technologies Inc, San Diego, CA) 1:1 for 15 minutes to prevent adherence to the plate.

For intracellular staining, Golgi stop protein (Brefeldin A) (Sigma Aldrich, St. Louis, Missouri) was added to the cells three hours before harvesting. The cells were washed twice in PBS, 2% FBS and then stained for cell surface markers as above. The cells were
then permeabilized with 200μL fixation and permeabilization buffer (BD Biosciences, San Jose, CA). The cells were incubated for 20 minutes away from light. After permeabilization, the cells were washed with fixation and permeabilization wash buffer 10x (Diluted 1:9 with dH2O). The cells were spun down and the buffer decanted. The cells were re-suspended in 100μL PBS, 2% FBS and then antibodies to intracellular markers conjugated to fluorescent dyes (anti-TNF-α-PE) were added according to manufactures specification. The cells were incubated for 20 minutes at room temperature away from light. The cells were washed twice with up to 3mL PBS, 2% FBS twice. The cells were then fixed in PBS, 1% Para formaldehyde, 2% FBS. Differences in CD16 and CCR5 Cell surface expression between stimulated versus unstimulated cells were compared and considered significant by Student’s t-test if $p<0.05$. 
Specific Aim # 4: To characterize HIV-1 binding to activated monocytes Vs non-activated monocytes using LAI and p89.6 viral strains.

Hypotheses: HIV-1 binding to activated monocytes (CD14+/CD16+) will be higher than to non-activated monocytes (CD14+/CD16-).

Rationale: There is increased surface expression of CD16 and CCR5 on monocytes during activation [16, 55]. This may enhance binding of HIV-1 strains that preferentially use CCR5 HIV co-receptor.

2.7 Experimental Design for Specific Aim # 4

Activated monocytes/macrophages were isolated and purified from IL-10 primed and LPS stimulated fresh whole blood. Viral binding assays as outlined in Specific Aim #1 were then performed on the activated cells.

2.7.1 HIV-1 binding to activated monocytes using LAI and p89.6 strains.

Fresh activated monocytes obtained from Ficoll, Percoll and Easysep Human Enrichment ‘without CD16 Depletion’ after IL-10 whole blood priming followed by LPS were suspended in RPMI 200μL RPMI supplemented with 0.2% normal human serum, 100 units/mL penicillin/Streptomycin. The cells were cultured in a 96 well polypropylene plate at 5 x 10⁵ cells/well and immediately exposed to HIV-1. The cells were exposed to
2ng LAI and p89.6 in duplicate as there weren't enough cells for a triplicate set up. Negative controls were also set up without virus exposure. The cells were incubated for 1 hour to allow for viral binding. The cells were thoroughly mixed with a transfer pipette and then incubated at 37°C, 5% CO₂ in the incubator for one hour. The plate was spun down at 1200rpm for 10 minutes at room temperature and 100µL of supernatant was removed using a Pasteur pipette. 200µL PBS was added to wash. The plate was spun down again to wash and the whole wash procedure was repeated three times. After the last wash, PBS was completely removed and 350µL lysis buffer was added to lyse the cells. The cell lysates were transferred into 1.5 mL RNase free tubes. The lysis was repeated and the lysate were pooled to respective tubes. The lysate were either frozen down at -80°C or RNA was immediately extracted. RNA concentration and purity was measured by Nanodrop Spectrophotometer. 75ng of RNA was used to make cDNA.

Viral binding capacity was assayed by RT-PCR using HIV gag and β-actin primers with appropriate positive and negative control RNA. Amplified fragments were resolved on 2.5% agarose gels and analyzed by densitometry. From the scanned gels, the ratio of HIV gag light units/β-actin light units was compared between the two virus strains. The HIV gag light units/β-actin light units for activated monocytes was also compared to the HIV gag light units/β-actin light units for non-monocytes. Differences in binding capacities between each of the two groups were considered significant by Student's t-test if \( p < 0.05 \). Significance between different viral strain treatments were analyzed by one-way ANOVA and were considered significant if \( p < 0.05 \). The statistics were performed using
SigmaStat 3.0 (SPSS Inc) of the duplicate experiments with the average ratio of HIV 
gag/β-actin.

<table>
<thead>
<tr>
<th>Gene</th>
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<tbody>
<tr>
<td>β-actin For</td>
<td>CCTGCCTTTGCGGATCC</td>
</tr>
<tr>
<td>β-actin Rev</td>
<td>GGATCTTCAGAGGTAGTCAGTC</td>
</tr>
<tr>
<td>HIV gag For</td>
<td>ATCAAGCAGCCATGCAAATGT</td>
</tr>
<tr>
<td>HIV gag Rev</td>
<td>CTGAAGGGTACTAGTAGTTCCCTGCTATATC</td>
</tr>
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Table 1 Oligonucleotide Primers used for PCR

Sequences for forward (for) and reverse (rev) primers are given in the 5' to 3' direction. The sequences for 
β-actin primers were obtained from Ziemssen, T., et al [36] where as the sequences for HIV gag primers were obtained from Klein et al [35].
CHAPTER 3.  RESULTS

The overall goal of this Master's Thesis Project is to determine the relative infectivity to HIV-1 of non-activated monocytes/macrophages (CD14+/CD16+) compared to activated monocytes/macrophages (CD14+/CD16+). The results for the Master's Thesis are summarized below for the proposed hypotheses and demonstrate accomplishment for the specific aims. Briefly, that the results demonstrate: 1) HIV-1 bound to unstimulated peripheral blood mononuclear cells (PBMCs), representing mainly lymphocytes, was higher for an HIV-1-lymphocyte-tropic viral strain (LAI) compared to monocytes; 2) Similarly for an HIV-1-macrophage-tropic viral strain (p89.6), more virus was bound to monocytes compared to PBMCs; and 3) Preliminary data suggest that activated monocytes, primed and stimulated with IL-10 and LPS, respectively, bound more virus compared to non-activated monocytes.

Specific Aim # 1: To evaluate HIV-1 binding to PBMCs compared to monocytes using LAI and p89.6 HIV-1 strains.

3.1 Evaluation of HIV-1 binding to PBMCs compared to monocytes using LAI and p89.6 HIV-1 strains.

T-lymphocyte- tropic LAI tended to have a higher viral binding to PBMCs which largely comprise lymphocytes compared to monocytes. The converse was true for macrophage-tropic p89.6 HIV strain. p89.6 had a higher viral binding to monocytes compared to PBMCs. Viral binding tended to increase in a dose dependent manner. To determine optimal viral concentrations (p24 units) for the viral binding assays, viral titrations were
carried out using both viral strains. Initially, PBMCs were isolated and analyzed by flow cytometry. Flow cytometry results were displayed in dot-plot format showing side scatter (separation based on complexity and granularity) and forward scatter (separation based on size of cells), Figure 1. In Figure 1A, the PBMCs were in the center of the scatter plot whereas the monocytes were located to the upper-right portion of the graph, Figure 1C. The size and scatter differentials correspond to the characteristics of monocytes which are more complex and larger than PBMCs. Figure 1 shows an example of flow cytometry results from anti-CD14-FITC stained cells following PBMC isolation. The graph shows that the PBMCs were comprised of 14.8% monocytes, Figure 1A. However, there was some non-specific binding from the Isotype control of 0.3%, Figure 1B, and when this factor was removed; the percentage of monocytes obtained was 14.1%, Figure 1C. After gating on the monocyte fraction as shown by the red lines, it was found out that the gate was comprised of 84.4% pure monocytes, Figure 1D.
Figure 1. Flow Cytometry Analysis of Isolated PBMCs using Ficoll.  A) Cellular debris is represented in the lower left region, the middle region shows lymphocytes (T cells, B cells and NK cells) and the upper right region shows monocytes; B/C) The Ficoll procedure yielded PBMCs that were comprised of 14.8 monocytes. Non-specific binding accounted for 0.3%; when this factor was removed, monocyte percentage was 14.1%. D) After gating on the monocyte fraction as shown by the red lines, it was found out that the gate comprised 84.4% pure monocytes.
To obtain pure monocytes for the viral binding assays, an aliquot of PBMCs was further purified by Percoll and Dynal magnetic bead. The Percoll procedure yielded 20.9% monocytes from PBMCs, Figure 2A. The monocyte fraction, Figure 2B, were gated and comprised more than 84.5% CD14+/CD16- cells and 5.44% were double-positive for CD14 and CD16.

**Figure 2. Flow Cytometry Analyses of Isolated Monocytes from PBMCs using Percoll.** A) Forward- and side-scatter plots show debris in the left lower region, the middle section shows lymphocytes (T cells, B cells and NK cells) and the upper right shows monocytes. Monocytes comprised 20.9% of the PBMCs; B) The monocyte fraction comprised more than 84.5% CD14+/CD16- cells and about 5.44% were double-positive for CD14 and CD16.
The Dynal magnetic bead selection procedure yielded more than 98% pure monocytes with CD14⁺/CD16⁻ phenotype as shown in Figure 3A. The gated monocytes were found to be 93% CD14-positive, Figure 3B. On average, approximately $2 \times 10^6$ monocytes were obtained from $10 \times 10^6$ PBMCs.

![Flow Cytometry Analysis of Isolated Monocytes using Dynal Magnetic Beads](image)

**Figure 3. Flow Cytometry Analysis of Isolated Monocytes using Dynal Magnetic Beads.** A) Relatively pure monocytes (98%) based on forward- and side-scatter; B) Gated monocytes were found to be 93% CD14-positive.

Determination of the optimal number of washes to remove unbound virus was accomplished by assaying HIV gag in the cDNA from RNA isolated from the supernatant washes following controlled viral binding experiments. As expected, HIV gag was detected in the initial supernatant as well as in the first wash; Figure 4, demonstrating that unbound virus was present in the supernatant and the first wash. No HIV gag detected in the second through fifth washes, Figure 4, suggesting that that cell-
free virus was removed completely by the second wash. Thus for the viral binding experiments, three washes were performed after viral binding to completely eliminate unbound virus.

![Image](image_url)

**Figure 4. Ethidium bromide-Stained Gel of HIV-1 Gag PCR Results for Optimizing Washout.** Example of duplicate experiments using HIV-1 viral stock p89.6 (82.143ng/μL p24 units) with PBMCs and monocytes. An aliquot (800μL) of the supernatant before washing was saved for RNA extraction. The cells were washed five times and each wash was saved for RNA extraction. Lane 1 contained viral supernatant (before washing) and lanes 2 to 6 contained the five washes. Lane 7 was the positive control and lane 8 was the negative control. HIV-1 gag was detected in lanes 1 and 2 near the expected 152bp size.
To determine how much virus to add to cells for the viral binding assays, viral titration experiments were performed using different amounts of HIV-1 viral stock p89.6 (82.143ng/mL p24 units). HIV-1 binding was assayed by RT-PCR and densitometry. The band intensity increased as more virus was added and reached saturation when 8ng p24 units of virus were added. Thus, the key experiments were designed to prevent saturation of the cells by virus, i.e. 2ng p24 units of HIV-1.

Assessment of HIV-1 binding was carried out by assaying RNA recovered from cells exposed to cells. β-actin PCR was used to assess the quality of the cDNA obtained as well as to normalize the results of the assays. β-actin PCR primers used were designed to amplify human cDNA and not genomic DNA (gDNA). The primers amplify a 626bp, Figure 6 for PBMCs, Figures 5 for monocytes. ACH-2 cell line cDNA was used as a positive control whereas PBMC DNA, which cannot be amplified by the selected primers, was used as a negative control.
Figure 5. Ethidium bromide-Stained Gel of β-actin PCR Results from Monocytes. Lanes 1-6 represent duplicate monocytes exposed to virus; Lane 7 negative control (PBMC DNA); Lane 8 Positive control (ACH-2 cDNA). The bands were within the expected range of 626bp.

Figure 6. Ethidium bromide-stained gel of β-actin PCR results for PBMC samples (lanes 1-6), negative control (PBMC DNA) in lane 7, and Positive control (ACH-2 cDNA) in lane 8. The bands were within the expected range of 626bp.
HIV-1 gag PCR was performed on all the samples. Amplification was obtained in all the samples where HIV-1 was added, Figure 7 (PBMCs) and Figure 8 (monocytes). The gel pictures were scanned using the Lumi-imager to compare and quantify band intensities as shown in Figure 9. The band intensity was correlated with the amount of virus bound.

**Figure 7. Ethidium bromide-Stained Gel of HIV-1 Gag PCR Results from PBMCs.** Using HIV-1 viral stock p89.6 (82.143ng/mL p24 units), 1ng p24 units of virus were added in lanes 1 and 2, 2ng in lanes 3 and 4, 3ng in lanes 5 and 6, 8ng in lanes 7 and 8, and 100μL in lanes 9 and 10. No virus was added in the negative controls (lanes 11 and 12). The bands were in the expected 152bp range.
Figure 8. Ethidium bromide-Stained Gel of HIV-1 Gag PCR Results from Monocytes. Using HIV-1 viral stock p89.6 (82.143 ng/mL p24 units), 1 ng p24 units of virus were added in lanes 1 and 2, 2 ng in lanes 3 and 4, 3 ng in lanes 5 and 6, 8 ng in lanes 7 and 8, and 100 μL in lanes 9 and 10. No virus was added in the negative controls (lanes 11 and 12). The bands were in the expected 152 bp range.

Figure 9. Example of Lumi-imager Results for the Viral Titration Assay. Lumi-imaging of the PCR gel picture generated digital data that revealed that there was increasing intensity of the bands from lanes 2 to lane 8 (1 ng to 4 ng). There was saturation in lanes 9 and 10 (8 ng). It was concluded that 4 ng p24 units be the limit for subsequent viral exposures.
Viral binding results are calculated from the amount HIV gag detected (BLU) normalized to the amount of β-actin detected from each replicate. A comparison of viral binding between PBMCs and monocytes showed that LAI had a higher viral binding capacity to PBMCs than monocytes. p89.6 had a higher viral binding capacity to monocytes than to PBMCs, Table 2 and graphed in Figure 10. Table 2 shows Densitometry results in Biological Light Units (BLU). Results for negative controls without virus exposure are not shown as the figures for gag were zero after background correction.

Viral binding appeared in a dose dependent manner. The more virus was added, the more was bound until there was saturation. 2ng p24 units of the viral stocks for LAI and p89.6 were the optimal amount of virus to add during viral binding. LAI viral binding increased as more virus was added to PBMCs up to 8ng p24 units but reached saturation at 4ng p24 units in monocytes. The converse was true for p89.6; more virus was bound as more was added but showed saturation in PBMCs as shown in figure 11. The saturation kinetics was similar to those obtained from LAI.
Table 2. LA1 and p89.6 Binding Assay

<table>
<thead>
<tr>
<th></th>
<th>LA1 1</th>
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<th>LA1 3</th>
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<td>LAI</td>
<td>p89.6</td>
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<tr>
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<td>0.386311</td>
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</table>

Figure 10. LA1 and p89.6 Binding Assay. PBMCs and monocytes were exposed to 2ng p24 units LA1 (A) and p89.6 (B) all in triplicate. Viral binding was assayed by RT-PCR and densitometry. There was a trend for LA1 to bind preferentially to PBMCs compared to p89.6 preferentially to monocytes.
Figure 11. LAI and p89.6 Binding Assay. PBMCs and monocytes were exposed to 2ng p24 units LAI (B). PBMCs and monocytes were exposed to 0.7ng, 2ng and 4ng p24 units p89.6 (A). Viral binding was assayed by RT-PCR and Densitometry. LAI tended to bind preferentially to PBMCs where as p89.6 tended to bind more preferentially to monocyte. N=3.

The significance of the results from Specific Aim # 1 is that monocytes bind more macrophage-tropic HIV strains. Monocytes have CCR5 surface expression. The higher HIV binding would be expected especially for macrophage-tropic virus strains that use the CCR5 co-receptor.
**Specific Aim # 2:** To evaluate CD16 expression on monocytes/macrophages grown in culture over a seven day period without stimulation and to characterize initial CD16 surface expression on cultured monocytes. To develop a non-activating culture system for monocytes

### 3.2 CD16 Expression Assay

CD16 expression initially on Day 0 in culture showed that 2.2% of monocytes expressed CD16, Figure 12. The level of CD16 expression dropped from day 1 to day 3 and was maintained up to day 7, Figure 12. The decrease is the number of CD16\(^+\) cells may be attributed to dying of cells in culture which was observed by both trypan blue and flow cytometry staining. The cultured cells did not show an increase in the level of CD16 expression over the seven day culture period, Figure 12. There was an increase in CD16 in the second experiment that had cells supplemented with M-CSF but the level sharply declined over the seven day culture period, Figure 12.

The results show that there was no activation of monocytes under the conditions set up for culture. However, due to the amount of cell death by day three, viral binding assays were conducted the same day cells were isolated. This was to make sure that the assays were performed on fresh and viable cells. Also, some of the HIV co-receptors like CCR5 have been observed to decrease surface expression after culturing cells. Immediate exposure of the cells to the virus strains eliminated a lot of variables in the assays.
The significance of these results from Specific Aim # 2 is that the initial CD16 expression on monocytes was determined. The amounts of CD16 expression were measured over seven days to see if there was any increase without any stimulation. There was no increase in CD16 surface expression over the entire period. This was important to note as viral binding to non-activated monocytes (without CD16) was done without cells being activated.

**Figure 12. CD16 Expression on Cultured Monocytes.** 2x10^5 Monocytes were cultured in RPMI over seven days. The cells were stained with CD14-FITC and CD16-PE, fixed and run on a FACS Aria flow cytometer. Expression of CD16 was noted over a seven day period; with little increase in expression of CD16.
Specific Aim # 3: To activate monocytes in vitro using lipopolysaccharide (LPS)

To develop an artificial system for activating monocytes

3.3 Whole Blood Stimulation

PBMCs and monocytes separated from whole blood primed with IL-10 and stimulated with LPS were stained with anti-CD14-FITC, anti-CD16-Alexa flour 647, anti-CCR5-APC Cy7 and anti-TNF-α-PE. The cells were analyzed by FACS Aria flow cytometry, Figure 13. High side scatter cells were gated on as the monocyte population. CD14 expression dramatically reduced in the monocytes population. Only 3.9% of the cells in the high side scatter expressed CD14, Figure 13 B. Also, there was not significant increase in TNF-α expression. However, there was a significant increase in CD16 expression (8.4%) and CCR5 expression (55.9%).

Monocytes where isolated from IL-10 primed and LPS stimulated whole blood by Ficoll, Percoll and Easysep monocyte enrichment. To evaluate activation, high side scatter cells (monocytes/macrophages) were gated on as shown in Figure 14 A. CD14 expression was reduced though the high side scatter cells were 93.5% CD14 as shown in Figure 14B.

LPS stimulation activated monocytes as shown by increased CD16 surface expression. This was also followed by increased surface expression of CCR5. CCR5 is an HIV coreceptor used by macrophage-tropic strains.
Figure 13. Flow Cytometry Analysis of Whole Blood Stimulation. PBMCs were isolated by Ficoll from stimulated whole blood. A) Monocytes gated on high side scatter. B) CD14 expression dramatically reduced in the monocytes population. Only 3.9% of the cells in the high side scatter expressed CD14; no significant increase in TNF-α expression; significant increase in CD16 expression (8.4%) and CCR5 expression (55.9%).
Figure 14. Flow Cytometry Results of Monocytes Isolated using Easysep Human Monocyte Enrichment. Monocytes were isolated from IL-10-primed and LPS-stimulated whole blood by Easysep. A) To evaluate activation, high side scatter cells (monocytes/macrophages) were gated as noted; B) CD14 expression was reduced though the high side scatter cells were 93.5% CD14.

The significance of the results from Specific Aim #3 is that activated monocytes with increased CCR5 surface expression could imply that higher HIV binding on activated monocytes may occur compared to non-activated monocytes. The higher HIV binding was expected especially for macrophage-tropic virus strains that use the CCR5 co-receptor.
Specific Aim # 4: To characterize HIV-1 binding to activated monocytes Vs non-activated monocytes using LAI and p89.6 viral strains.

3.4 Characterization of HIV-1 Binding to Non-activated Monocytes versus Activated Monocytes

Activated monocytes obtained by Easysep Human Monocytes Enrichment without CD16 depletion were exposed separately to 2ng LAI and p89.6 viral strains. Exposure was restricted to one hour to allow for viral binding. For technical reasons, viral binding was set up in duplicate with one negative control without virus exposure.

Viral binding was measured by RT-PCR and densitometry. The HIV-1 gag/β-actin ratios obtained where compared to those obtained from non-activated monocytes viral binding assays. The results obtained (Table 3) show an increase in viral binding in activated monocytes compared to non activated monocytes, Figure 15. The results were however not statistically significant due to the few number of replicates and since only one run of the assay was performed. However, the trend set by the assays performed is that there is increased viral binding to activated monocytes and the M-tropic viral strains have a higher binding capacity to activated monocytes than T-tropic strains.
Table 3. Lumi-imager Results for LAI and p89.6 Binding to Activated Monocytes

<table>
<thead>
<tr>
<th></th>
<th>LAI (Gag/β-actin BLU)</th>
<th>p89.6 (Gag/β-actin BLU)</th>
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<tr>
<td>Rep #1</td>
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<tr>
<td>Rep #2</td>
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Figure 15. LAI and p89.6 Binding to Activated Monocytes. Activated monocytes obtained from whole blood stimulation with LPS were exposed to 2ng p24 units of LAI and p89.6 HIV-1 strains. A trend for activated monocytes having a higher HIV-1 binding capacity compared to non-activated monocytes is shown. Viral binding between the two viral strains was however not statistically significant. N=2, p=0.667.
HIV gag/β-actin BLU from the LAI and p89.6 binding to activated monocytes (Table 3) were compared to experiments from non-activated monocyte, Figure 16. Viral binding to activated monocytes trended to be higher compared to non-activated monocytes for the two viral strains. The results however were not statistically significant between the treatment groups as analyzed by one way ANOVA, p>0.05.

![Graph showing LAI and p89.6 Binding to Activated Monocytes Versus Non-activated Monocytes](image)

**Figure 16. LAI and p80.6 Binding to Activated Monocytes versus Non-Activated Monocytes.** Trend for viral binding to activated monocytes to be higher compared to non-activated monocytes (n=3, non-activated monocytes; n=2, activated monocytes), p=0.362.

The significance of these results from Specific Aim # 4 lies in that increased viral binding of activated monocytes could allow the cells to be more permissive to HIV-1 infection. This leads them to harbor more proviral DNA, which in PBMCs, has been associated with HAD.
HIV-1 bound to unstimulated peripheral blood mononuclear cells (PBMCs), representing mainly lymphocytes, was higher for an HIV-1-lymphocyte-tropic viral strain (LAI) compared to monocytes. Similarly for an HIV-1-macrophage-tropic viral strain (p89.6), more virus was bound to monocytes compared to PBMCs; and preliminary data suggest that activated monocytes, primed and stimulated with IL-10 and LPS, respectively, bound more virus compared to non-activated monocytes.

The significance of the results is that monocytes bind more macrophage-tropic HIV strains. The results demonstrating that activated monocytes have an increase in CCR5 surface expression could account for macrophage-tropic virus having a higher binding capacity to the activated monocytes. The higher HIV binding would be expected especially for macrophage-tropic virus strains that use the CCR5 co-receptor. T-tropic HIV strain, LAI, also bound to monocytes. The implication of binding of the different HIV-1 strains would be translated to monocytes being more permissive to infection. HIV-1-infection in monocytes leads to chronic infection as HIV is not cytopathic to monocytes. Monocytes would then harbor more HIV proviral DNA which has been associated with HAD. It is expected that the high HIV DNA detected in PBMCs in persons with HAD is in the monocyte fraction. This phenomenon also suggests that peripheral events outside the central nervous system may play a role in neuropathogenesis of HIV-1.
Initial CD16 expression on monocytes was quantified and ranged from 2 to 5%. The amounts of CD16 expression were measured over seven days to see if there was any increase without any stimulation. There was no increase in CD16 surface expression over the entire period. It was expected that monocytes placed in culture would become activated. Media components as well as cultureware have been shown to activate monocytes. This was important to note as viral binding to non-activated monocytes (without CD16) needed to be done without cells being activated. Monocytes do not bind to the cultureware used, polypropylene and Teflon, and may have contributed to why they did not become activated [59].

Whole blood stimulation with LPS after IL-10 priming yielded activated monocytes with a CD14+/CD16+ phenotype. The amount of activation markers assayed (CD16 surface expression and intracellular TNF-α) was lower than expected. However, there was a dramatic increase in CCR5 surface expression after the monocytes become activated. Monocytes have been shown to increase surface expression of CCR5 during inflammation or HIV-1 infection [49-51].

Viral binding to activated monocytes was higher than to non-activated monocytes. It has been shown that individuals with HAD have higher percentages of circulating activated monocytes/macrophages (CD14+/CD16+) [8, 26-28]. Having shown that activated monocytes have a higher HIV-1 binding capacity, they are more permissible to infection and harbor more proviral DNA. It is expected that the observed high HIV proviral DNA in PBMCs is in activated monocytes. HIV-1-infected activated monocytes also have
increased trafficking into the CNS. By trafficking into the CNS they transport HIV-1 by ‘Trojan horse’ mechanism. Monocytes in the CNS differentiate into long lived microglia. HIV-1-infected microglia serve as reservoirs infection transmitting the virus to other cells such as oligodendrocytes, astrocytes and neurons. Activated monocytes thus play direct or indirect role to induce neuronal injury. Direct neuronal toxic mechanisms involve release of viral molecules (gp120, Tat, Vpr) shed by HIV from infected monocytes/macrophages and microglia that directly injure neurons. Indirect mechanisms involve influx of activated monocytes/macrophages that activate perivascular microglia to release proinflammatory cytokines and chemokines (interleukin-1β and tumor necrosis-α) and other excitatory mediators (TRAIL, glutamate, quinolinic acid) that induce neuronal apoptosis [2, 34-36]. Proinflammatory cytokines increase expression of adhesion factors on brain endothelial cells enabling further transmigration of infected and activated monocytes/macrophages into the brain [10, 37]. Furthermore, activation and excitation of astrocytes modifies the permeability of the blood-brain barrier and promotes the migration of more monocytes in the brain [10].

The significance of the Master’s Thesis Results is consistent with the clinical finding where higher amounts of HIV DNA in PBMC are found in patients with HAD. The results demonstrated that the monocyte fraction and particularly the activated monocyte fraction binds more macrophage-tropic HIV strains that use CCR5 co-receptor. Whether this finding leads to permissive infection remains to be shown. High HIV DNA in PBMC has been correlated to HAD linking peripheral events outside the central nervous system to play a key role in the development of HAD.
4.1 CONCLUSION
The high HIV DNA observed in PBMC is in monocytes particularly the activated monocyte fraction. Activated monocytes have a higher binding capacity to macrophage-tropic HIV strains and are thus more permissive to infection. The implication of this finding lies in the potential role in the neuropathogenesis of HIV-1. If validated, eradication of HIV-1 in activated monocytes may be considered a key component as a therapeutic target to prevent or treat HAD.

4.2 FUTURE DIRECTION
The results from the Master Thesis partially fill a void in our understanding of HIV-1-associated neurocognitive problems. However, the results also lead to new questions and ideas for consideration. Characterizing HIV-1-binding and infection of activated monocytes/macrophages (CD14+/CD16+) compared to non-activated monocytes/macrophages (CD14+/CD16-) remains to be completed. Since viral binding is part of the initial sequence of events leading to viral infection, characterizing infection of activated monocytes/macrophages could be a natural progression of future studies.
REFERENCES


