The Role of CD4 polymorphism on HIV infection and Disease progression

By

Julius Otieno Oyugi

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

Doctor of Philosophy

Department of Medical Microbiology
University of Manitoba
Winnipeg

Copyright © 2009 by Julius O. Oyugi
NOTICE:

The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

AVIS:

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.
The Role of CD4 polymorphism on HIV infection and Disease progression

BY

Julius Otieno Oyugi

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of
Manitoba in partial fulfillment of the requirement of the degree

Of

DOCTOR OF PHILOSOPHY

Julius Otieno Oyugi © 2009

Permission has been granted to the University of Manitoba Libraries to lend a copy of this
thesis/practicum, to Library and Archives Canada (LAC) to lend a copy of this
thesis/practicum, and to LAC's agent (UMI/ProQuest) to microfilm, sell copies and to
publish an abstract of this thesis/practicum.

This reproduction or copy of this thesis has been made available by authority of the
copyright owner solely for the purpose of private study and research, and may only be
reproduced and copied as permitted by copyright laws or with express written
authorization from the copyright owner.
Abstract

Since the first reported cases of AIDS in 1981, to date there is no vaccine available to prevent HIV infections and the anti-retroviral drugs available can only prolong the lives of those infected. Sub-Saharan Africa, with about 10% of the global population, harbors over 65% of all HIV infected individuals. Many factors including host genetics have been documented that helped in the spread of HIV in this region. A Single Nucleotide Polymorphism (SNP) on CD4 found at higher prevalence among people of African descent is a possible host genetic factor that could explain the dramatic spread of HIV in this region. We hypothesize that a SNP encoding an amino acid change in the third domain of CD4-Trp240(868T) may alter its tertiary structure thus changing its functions with respect to HIV-1 infection or signal transduction. We investigated these possibilities by testing the susceptibility of a T-cell line, A2.01, transfected to express similar levels of either phenotype (A2.01/868T) or wild-type CD4(A2.01/868C), to both laboratory-adapted HIV viruses and clinical HIV isolates. We also investigated whether CD4-specific signal transduction is altered in cells with A2.01/868T cells by cross-linking CD4 with anti-CD4 monoclonal antibody. A2.01/868T cells were found to be more susceptible to infection with both X4-tropic virus and clinical HIV isolates relative to cells that express wild-type CD4. Subsequent infection of A2.01/868T and A2.01/868C cells with a CD4-independent virus showed no difference in the cell lines' ability to support HIV-1 replication. This demonstrates that differences in the infections observed when using a CD4-dependent virus are due to the CD4 isotype and not the cell lines' ability to support HIV-1 replication. Although the CD4-Trp240(868T) was found to bind gp120 of HIV-1 with low affinity, cross-linking of these cells with anti-CD4 demonstrated that signal transduction was enhanced, immune activation markers were elevated and apoptosis occurred at a higher rate in these cells than those with wild-type
CD4 phenotype. This implies that the ability of cells expressing CD4-Trp240(868T) to become activated more easily could be a factor in the increased susceptibility to HIV-1 infection observed. In conclusion, in addition to confirming that CD4 SNP is a risk factor with respect to HIV infection and disease progression, this study has provided insight into the molecular basis for these risk factors. Those who have this polymorphism have CD4+T cells that have increased susceptibility to HIV-1 and upon infection they lose these cells more rapidly by either apoptosis or activation induced cell death.

There are three fundamental significant findings from this study. First, CD4 polymorphism is highest among the Africans, the same population with the highest HIV-1 prevalence globally. This study together with the previous epidemiology studies, confirm that CD4 SNP is one of the host factors that could have contributed to the rapid spread of HIV in Sub-Saharan Africa. Second, given that there is no effective vaccine for prevention of HIV-1 infection, there is need to improve on the currently available anti-retroviral drugs. The finding that gp120 of HIV binds to CD4-Trp240(868T) with low affinity is an important observation and could be used to help in designing better anti-retroviral drugs especially the fusion inhibitors. Lastly, the finding that individuals with CD4 SNP progress rapidly to AIDS due to either apoptosis or activation induced cell death should be used to investigate further how these mechanisms could be inhibited with the view of slowing disease progression.
Dedication

I dedicate this thesis to three most important people in my life. My dedication goes to my wife who gave-up her career to sacrifice everything so that I could achieve my ambition of attaining the highest level of academic education. My dedications also go to my Mother, the late Paskalia Awuor Oyugi who consistently encouraged me to work hard in school and to always be on top of everyone even though she never saw the inside of a classroom. I also dedicate this work to my wonderful children for being there for me when I needed them most.
Acknowledgements

I would like first to thank my supervisor for all his help during my graduate studies. I honestly admit that you provided me with all the necessary tools that a student would need for success in his studies. I remember how you always had not just a solution to academic problems but solutions. Indeed, I cannot remember any single day that I walked into you office with a problem and never came out with solutions to the problem. I honestly appreciate the fact that you were always there whenever I needed help with project.

I would also like to thank my committee members, Dr. J. Rempel, Dr. F. Plummer, Dr. A. Michele and Dr. J. Berry for their valuable support during my committee meetings. Your questions and suggestions during my committee meetings help me a great deal in shaping the direction of my project.

My thanks also go to Dr. Blake Ball and to the head of the Department of Medical Microbiology, Dr. Joanne Embree for always accepting to be my referee at short notice. Your letters of support were instrumental in helping me get various fellowships over the last five years.

Great thanks also go to fellow students in Medical Microbiology both present and past. My studies would not have been complete without your help. I learnt a lot about HIV-1 from you through seminars, literature late review and student presentations. In particular, my great thanks go to Hezhao Ji, Jennifer Juno and Jill Waruk for always helping me without seeing obstacles or barriers. To members of the Fowke laboratory team, thanks for your help with everything.

Asante sana to Judie Alimonti, Paul McLaren, Sandra Koesters, Françoise Vouriot, Catherine Card, Yoav Keynan, Jennifer Juno and Stephen Wayne. Thanks too to John Rutherford and Leslie Slaney for teaching some of the techniques that were useful for my project.
To all University of Manitoba, Department of Medical Microbiology staff, thank you so much for your support. You provided a warm and friendly environment which made it easy for me to always get whatever I wanted within the shortest time possible.

Lastly, I would like to thank God for giving good health throughout my study period and for being there for me whenever things seemed so bad.
1.0 Introduction
1.1. The discovery of AIDS 1.
1.2. The origin of HIV 2.
1.3. HIV/AIDS a global problem 4.
1.4. HIV virology
   1.4.1. Classification and structure 6.
   1.4.2. Replication 10.
   1.4.3. Transmission 14.
1.5. HIV disease course and pathogenesis
   1.5.1. Acute stage 16.
   1.5.2. Chronic stage 18.
   1.5.3. AIDS Stage 19.
1.6. HIV pathogenesis 22.
1.7. Susceptibility to HIV-1 infection and disease progression to AIDS 23.
   1.7.1. Viral Factors
      (i) HIV subtypes 23.
      (ii) Viral Escape 25.
      (iii) Viral Attenuation 26.
      (iv) Viral tropism or phenotypic switch. 26.
   1.7.2. Host Factors
      (i). Genetic factors that regulate immune responses 27.
      (ii). Genetic factors that modulate HIV entry 28.
1.9. CD4 membrane glycoprotein 31.
   1.9.1. Structure and functions 31.
   1.9.2. The function of CD4 as a signal transduction receptor 34.
   1.9.3. The role of CD4 as a receptor to HIV 38.
   1.9.4. The role of CD4-gp120 interaction in HIV-1 immunodeficiency 38.
1.10. CD4 polymorphism 39.
2.0. Hypotheses and specific objectives

2.1. Study Rationale
2.2. Hypotheses
2.3. Specific Objectives

3.0. Materials and Methods
3.1. Source of Biological Materials
3.2. General Chemicals.
3.3. Culture Reagents
3.4. Reagents for specific methods
   3.4.1. Reagents for signal transduction studies
   (i) Western blot Assay
   (ii) Flow Cytometry Assay
   (iii) Luminex Assay.
   3.4.2. Reagents for HIV-1 infection studies
   (i). Reagents for propagation of HIV viral stocks
   (ii). HIV-1 p24 ELISA
   (iii). Luciferase assay
   3.4.3. Reagents for Binding Affinity studies-ELISA
3.5. Equipment used
3.6.1. Cloning of wild type and mutant CD4 genes
3.6.2. Transfection of A2.01 cell line with CD4 868C and 868T genes
3.6.3. Confirmation of the CD4 868C and 868T genes in the A2.01 cells
3.6.4. DNA Isolation
3.6.5. CD4 sequence analysis confirmatory Assay
3.6.6. Purification of anti-CD4 (SIM4)
3.6.7. Staining A2.01/868T and A2.01/868C cells for CD4 expression using anti-OKT4
3.6.8. Isolation of PBMCs from Whole blood
3.7. Specific methods
3.7.1. Binding affinity studies
   (i). Experiment set-up
   (ii). Binding affinity studies-ELISA method
   (iii). Binding affinity studies-Flow cytometry method
3.7.2. HIV infection studies
   (a). Infection using CD4 dependent HIV-1 viruses
      (i). Generating HIV stocks
      (ii). Calculation of virus titres
      (iii). HIV-1 infections experiment set-up
      (iv). HIV-1 p24 ELISA
      (v). Detection of intracellular HIV p24 ELISA by flow cytometry
   (b). Infection using CD4-independent virus
      (i). Luciferase assay-experiment set-up
      (ii). Luciferase assay-sample preparation and analysis
3.7.3. Signal Transduction studies
(i) Western Blot
(ii) Luminex assay
(iii) Flow-cytometry

4.0. Statistical calculation

5.0. Results
5.1. Developing cell lines for in vitro assay
5.2. CD4 polymorphism and susceptibility to HIV infection studies
   5.2.1. Evaluate susceptibility of CD4 868T cells to infection with
           CD4-dependent HIV-1.
   5.2.2. Assessing susceptibility of A2.01/868T and A2.01/868C cells.
           to CD4-independent HIV-1 virus
   5.2.3. Assessing susceptibility of persons with CD4 868T/C
           to HIV-1 infection
5.3 Assessing if there are differences in binding affinities between gp120
   and the CD4s on 868T and 868C cells.
5.4 The role of CD4 polymorphism on HIV/AIDS disease progression
   5.4.1. Assessing early signaling events in CD4 868T cells that may
           influence rapid cell death.
   5.4.2. Evaluating the impact of CD4 signaling in immune
           activation in CD4 868T cells

6.0 Discussion
6.1. CD4 polymorphism and HIV disease association
6.2. Possible mechanisms of CD4 868T for the risks of
   HIV infection and disease progression.
6.3. A2.01 cell line, a model for studying impact of CD4 SNP
   on CD4 functions
6.4. Cells expressing CD4-Trp240(868T) are more susceptible to HIV
   infection than 868C cells.
6.5. A higher CD4 binding affinity to gp120 is not a mechanism
   for increased susceptibility to HIV infection in individuals
   with 868T allele
6.6. AICD is a mechanism for rapid disease progression in persons
   with CD4 868T allele
6.7. gp120 induced signal transduction enhances HIV-1 replication
   in A2.01/868T cells
6.8. Immune Activation is a mechanism for rapid CD4+T cell loss
   in CD4 868T individuals
6.9. Apoptosis is a mechanism for CD4+T decline in individuals
   with CD4 868T allele

7.0. Conclusion and summary

8.0. Future Work
9.0. Study model
10.0 Reference
11.0 Abbreviations

List of Tables

Table 1: CDC classification of AIDS defining conditions  
Table 2: Sequencing primers  
Table 3: A layout of HIV-1 infection results for calculating TCID50  
Table 4: Tabulation of HIV-1 infection results for calculation of TCID50
List of Figures

Figure 1: The structure of human HIV-1 virus 8.
Figure 2: The genomic structure of HIV 9.
Figure 5: Structure of Human CD4 33
Figure 6a: The proposed CD4-specific signaling pathway 36.
Figure 6b: Regulation of p56lck enzymatic activity 37.
Figure 9: CD4 Expression on 868T, 868C before and after cell sorting 79.
Figure 10: Confirmation of 868T and 868C genes in the transfected A2.01 cells 81.
Figure 11: Staining A2.01/868T and A2.01/868C cells with anti-OKT4 83.
Figure 12: CD4-Trp240(868T) expressing cells are more susceptible to infection with HIV-1MB virus 86.
Figure 13: Determination of HIV-1 infected cells by flow cytometry 87.
Figure 14: Infection of A2.01, A2.01/868T and A2.01/868C (wt) cells with Kenyan primary HIV-1 viral isolate 89.
Figure 15: Infection of A2.01/868T and A2.01/868C cells with a CD4-Independent virus 92.
Figure 16: HIV-1 infection of PBMCs from donors with either CD4 868T/C and CD4 868C alleles 95.
Figure 17: Determination of binding affinity between gp120 and the two CD4 isoforms by ELISA method 98.
Figure 18: Determination of binding affinity between gp120 and the two CD4 isoforms by flow cytometry 99.
Figure 19: Determination of total phosphorylated lck between A2.01/868T and A2.01/868C cells by western blot. 103.
Figure 20: Determination of phosphorylated lck between A2.01/868T and A2.01/868C cells using luminex method 104.
Figure 21: Determination of the amount of phosphorylated tyr505 residue on lck between A2.01/868T and A2.01/868C cells 105.
Figure 22: Comparing of HLA-DR expression levels between un-stimulated and stimulated CD4 868T/C and CD4 868CC cells

Figure 23: Comparing CD69 expression on PBMCs from CD4 868T/C and CD4 868CC cells

Figure 24: Comparing the rate of apoptotic cell death activated A2.01/868T and A2.01/868C cells

Figure 25: The proposed model for CD4 868T increased susceptibility to HIV infection.

Figure 26: The proposed model for CD4 868T increase disease progression.
### List of Copyrighted Material

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 3</td>
<td>The life-cycle of HIV</td>
<td>13.</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Kinetic of HIV disease</td>
<td>20.</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Kaplan-Meier plot of time from enrollment to HIV-1 Seroconversion</td>
<td>41.</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Kaplan Meier plot of MCH CD4+ T-cell decline to less than 400 cells/cubic mm.</td>
<td>42</td>
</tr>
<tr>
<td>Figure 25</td>
<td>Mechanisms of gp120-induced killing</td>
<td>134.</td>
</tr>
</tbody>
</table>
1.0 Introduction

1.1 The discovery of AIDS

In June 1981, Centres for Disease Control (CDC) reported five cases of *Pneumocystis carinii* pneumonia (PCP), a disease that was almost exclusively limited to severely immunosuppressed patients (MMWR Weekly, 1981). The occurrence of pneumocystosis in previously healthy individuals without a clinically apparent underlying immunodeficiency and the development of rare opportunistic infections and cancers that seemed stubbornly resistant to any treatment among gay men in New York and San Francisco was indeed unexpected. These events marked the beginning of the search of an infectious agent that targets and destroys host immunity, causing opportunistic infections in otherwise healthy individuals. The disease was later named Acquired Immunodeficiency Syndrome (AIDS) in 1982.

Although the initial AIDS cases were reported in 1981, it is believed that this disease may have been in existence in Central Africa as early as 1959 (Zhu Tu, 1998). While the initial cases were mainly confined to gay men in the United States of America, it became clear that the disease had affected other population groups as well. For example, AIDS was reported in injection drug users (IDU) (Masur H, 1981), haemophiliacs (Davis KC, 1983, Poon MC, 1983), blood transfusion recipients (Curran JW, 1984), adults from central Africa (Piot P, 1984) and infants born to mothers who had AIDS (Oleske J, 1983). Additionally, evidence started coming out suggesting the presence of AIDS associated symptoms in different parts of the world. Between 1982 and 1985 a number of countries, including those in Europe (Vilaseca J, 1982, Rozenbaum W, 1982) and in Africa (Serwadda D, 1985), started reporting cases of individuals with AIDS.
As soon as it became evident that a new disease was spreading, the search for its causative agent also began. In May 1983, doctors at the Institute Pasteur in France reported that they had isolated the virus that causes AIDS and named it lymphadenopathy-associated virus (LAV) (Barre-Sinoussi F, 1983). A year later, Dr. Robert Gallo of the National Cancer Institute isolated a similar virus and named it HTLV-III. For sometime, both names were used to refer to a single infectious agent. Finally, in May 1986, the International Committee on the Taxonomy of viruses ruled that both LAV and HTLV-III were the same virus and named the virus Human Immunodeficiency Virus (HIV) (Coffin J, 1986). Recently, two French researchers Francoise Barre-Sinoussi and Luc Montagnier were awarded the 2008 Nobel Prize in Medicine for the discovery of Human Immunodeficiency Virus, or HIV.

1.2 The origin of HIV

Since the discovery of AIDS in 1981, the question of where HIV came from had not been fully answered. A number of theories were proposed for the origin of HIV. Such theories include; the hunter theory, the oral polio vaccine (OPV) theory and the conspiracy theory (Curtis T, 1992). To date, the hunter theory is considered the most convincing theory on the origin of HIV. According to this theory, AIDS is a zoonotic disease which came about as a result of transmission of different strains of simian immunodeficiency viruses (SIV) from primates to humans through primate blood getting into cuts or wounds on the hunter. Once inside the human body, SIV was able to adapt itself within its new human host and became HIV. Hunting for primate meat is a common practice in West Africa and given that primates are natural reservoirs for SIV it is possible that cross-infection of SIV into humans may have lead to the development
of the human form of the virus. There are three lines of evidence to support this theory. The first evidence relates to the similarities on the organization of the viral genome between the SIV and HIV viruses. For example, vpu one of the genes in lentivirus genome is only found in the genomes of both HIV-1 and SIVCPZ and not in other lentiviruses. Similarly, vpx another lentiviral gene is also only found in the genomes of Human Immunodeficiency Virus Type 2 (HIV-2) and SIVsm and not on the genomes of other lentiviruses (Huet T, 1990, Hirsch V, 1989). The sharing of these genes between primate and human lentiviruses suggests that both HIV-1 and HIV-2 could have arisen from different SIV strains. The second piece of evidence comes from phylogenetic studies of both human and primate lentiviruses. According to these studies, HIV-1 is closely related to SIVcpz from the chimpanzee sub-species Pan troglodytes troglodytes (Peeters M, 1989), while HIV-2 is closely related to SIVsm from sooty mangabeys (Hirsch V, 1989). The third line of evidence relates to geographical coincidence of certain strains of HIV-1 and their SIV counter-parts. HIV-1 groups M, N, and O have co-circulated in human populations in the same areas with chimpanzees infected with genetically closely related viruses suggesting geographical coincidence between SIV and particular HIV strains. HIV-1, a close relative to SIVcpz, is found in the geographic region corresponding to the range of P. troglodytes in west equatorial Africa (Simon F, 1998).

While these lines of evidence strongly support cross-species transmission of primate lentiviruses to humans, it is also known that the transmission from primate to humans was not a single event. Indeed, it is believed that SIV may have been introduced in human populations more than seven times (Chen Z, 1996, Gao F, 1999, Gao F, 1992). Furthermore, HIV-1 groups M, N and O are thought to represent separate SIVcpz transmission events to humans (Beatrice HH, 2000). There
is no specific time as to when these groups of HIV-1 were introduced in human population. One study even suggests that group M may have been introduced into human population as early as 1920 or 1930 (Hahn, BH, 2000).

1.3 HIV/AIDS a global problem

Although HIV may have originated from Central Africa, today the virus is found in all parts of the globe and has become a global health problem. Since its discovery in 1981, the HIV/AIDS pandemic has emerged to become one of the most important global public health challenges of all time. In more than 26 years since its discovery, HIV has spread relentlessly to virtually every country in the world. To date, it is estimated that more than 65 million persons worldwide have been infected with HIV, and more than 25 million have died of AIDS since the first reported cases of AIDS (Merson MH, 2006). Although AIDS is currently found in all corners of the globe, the hardest hit populations are those in developing countries. Part of the reason why these areas have suffered a great burden due to AIDS is because of a lack of resources to implement prevention intervention early in the epidemic. At the end of 2007, The Joint United Nations Programme on HIV/AIDS (UNAIDS) and World Health Organization (WHO) estimated that about 33 million people were living with HIV-1 globally, with 2.5 million new infections and 2.1 million deaths occurring in that year alone (UNAIDS, 2007). While sub-Saharan Africa account for about 10% of the world population, it harbors over 75% of all HIV/AIDS cases reported. Certainly, there a number of factors that may have contributed to the rapid spread of HIV-1 in this part of the word. These include widespread poverty, gender inequality, poor health systems, and factors associated with the host as well as the type of circulating viruses.
The impact HIV/AIDS has had in this region is well documented. According to the UNAIDS report of 2007, of the 2.1 million deaths reported globally, 1.6 million were from sub-Saharan Africa, making AIDS the single largest cause of death in the region (UNAIDS, 2007). As many adults die of AIDS and related diseases, they leave behind orphans. Consequently, the region has witnessed a dramatic rise in the number of orphans. Indeed, by the end of 2007, it was estimated that there were 11.4 million orphans due to AIDS in this region (UNAIDS, 2007). Additionally, the region has witnessed a dramatic reduction in life expectancy of up to 20 years since HIV was introduced in the area. The increased life expectancy witnessed in some of these countries due the introduction of antibiotics, vaccinations and clean drinking water has been completely reversed by HIV-1/AIDS. By killing young adults, HIV/AIDS has had far reaching effects on economies and societies in the region (UNAIDS, 2007).

In other regions of the globe, HIV/AIDS has slowed rather than reversed gains in life span. It is estimated that life expectancy in Cambodia is currently 4 years lower than it would have been without AIDS(UNAIDS, 2007).

Despite the availability of life saving antiretroviral drugs (ARVs), the development of inexpensive diagnostics tools and knowledge of prevention methods, HIV has continued to spread. The lack of an effective HIV-1 vaccine has meant an unabated spread of HIV in many parts of the world. However, in spite of these limitations, there are new and promising approaches to slow the spread of HIV-1. Adult male circumcision and pre-exposure antiretroviral prophylaxis in high-risk populations are some of the approaches being considered as prevention strategies. Indeed, recent reports by Bailey et al have shown that male circumcision can
significantly reduce the risk of HIV acquisition in young men in Africa (Bailey RC, 2007). While these approaches may slow down the spread of HIV in many areas, the only hope to stop the spread of this virus is to develop an effective vaccine or drugs that can treat AIDS. In order to develop these regiments, there is need to study all aspects of the HIV virus and its interaction with the human host.

1.4 HIV virology

The study of HIV encompasses understanding characteristics that makes it different from other viruses, how it replicates and the mechanisms of how it is passed from host to host.

1.4.1 Classification and structure

Human Immunodeficiency Virus belongs to the retroviridae family of viruses and is a member of a sub-family of retroviridae known as lentivirus (Sharp PM, 1994). The family of retroviridae is further divided into three sub-families; Alpharetrovirus, Betaretrovirus, Gammaretrovirus, Deltaretrovirus, Epsilonretrovirus, Lentivirus. As a lentivirus, HIV shares genetic and morphologic similarities to other animal lentiviruses (Gonda Wong-staal, 1985). These viruses primarily infect cells of the immune system, including T lymphocytes and macrophages (Levy JA, 1993) causing immunodeficiency in their hosts. Additionally, they cause slow, progressive wasting disorders, neurodegeneration and death (Haase AT, 1986). Although some lentiviruses have the ability to cause neurodegeneration in their hosts, their ability to cause these diseases is dependent in the host as well as the characteristics of the envelope of the infecting virus (Demuth, MS, 2000). All lentiviruses that cause neurodegenation infect monocytes by binding to their CD4. Since monocytes express low CD4 than T-Lymphocytes it is possible that the strains of
lentiviruses that infect these cells have higher envelope affinity to CD4 than the envelope from the same viruses that infect T-lymphocytes (Duenas-Decamp MJ, 2009). The primary characteristic of all retroviruses including lentiviruses is the possession of a reverse transcriptase enzyme that converts the single-stranded RNA genome into a double-stranded DNA copy. Additionally, lentiviruses also have a unique open reading frame (ORF) located between pol and env genes (Pyper JM, 1986, Sonigo P, 1985). In HIV virus, the ORF code for regulatory proteins that are not found in other retroviruses (Ratner L, 1985, Rushlow K, 1986). For example, vpu is only found in HIV and not in other lentiviruses.

Like other lentiviruses, HIV virus has a cylindrical core that encases two copies of positive single-stranded RNA and viral enzymes (integrase, protease, and reverse transcriptase). Each strand of RNA consists of about 9200 nucleotide bases and two other proteins, p6 and p7 (Fauci AS, 1988). The core is surrounded by lipid envelope (Figure 1). Env, pol and gag genes are other genes that are found in the genome of HIV virus but are also commonly found in the genome of other retroviruses.

At genetic level, the HIV provirus DNA consist of two long terminal repeat (LTR) elements at each end with nine viral genes located in between the LTRs (Figure 2). The gag, pol and env genes encode for structural proteins while tat and rev genes encodes for regulatory proteins that influence viral replication. The other remaining genes nef, vif, vpr and vpu encode for accessory proteins (Franza BR, 1987, Gallo R, 1988).
Figure 1: The structure of human HIV-1 virus. At structural level, HIV consists of two positive single stranded RNA and a reverse transcriptase enzyme enclosed in the matrix. The virus is enveloped and the envelope protein consists of a trans-membrane glycoprotein (gp41) and envelope glycoprotein (gp120).
Figure 2: The genomic structure of HIV. This figure shows the genomic organization of HIV-1 virus.
1.4.2 Replication

Like all viruses, HIV is an intracellular pathogen which requires the host cells' metabolic processes in order to replicate. The entire replication process involves many steps including binding and entry, reverse transcription, integration, transcription, translation, viral assembly and maturation. The first step in viral replication involves the viral gp120 binding to a host cell receptor, CD4. This host membrane glycoprotein is expressed on helper T lymphocytes, monocytes, B-lymphocytes and several other cell types (Dalgleish AG, 1984). The binding site of gp120 on CD4 has been mapped to the domain 1(D1) of the extracellular portion of CD4 (Arthos J, 1989). The binding of gp120 to CD4 induces conformational changes in gp120 that permit it to interact with a co-receptor (Feng Y, 1996, Wu L, 1996). The HIV co-receptors have been identified as members of the family of chemokine receptors. A number of these co-receptors have been identified, however, CXC chemokine receptor 4 (CXCR4) and CC chemokine receptor 5 (CCR5) are the most commonly used by X4-tropic and R5-tropic HIV-1 viruses respectively (Feng Y, 1996, Alkhatib G, 1996, Choe H, 1996). The binding of gp120 to a co-receptor triggers further conformational changes on the envelope protein exposing gp41 which forms a pre-hairpin intermediate (Jones PL, 1998). Consequently, the pre-hairpin intermediate exposes a fusion peptide of gp41 which the virus uses to bring about membrane fusion and entry into a host cell (Furuta RA, 1998).

After successful entry into a host cell, the virion's capsid is partially uncoated to form a ribonucleoprotein complex. Using the associated tRNA as a primer, the reverse transcriptase transcribes a minus sense cDNA strand from the viral genome. The viral RNA is then digested by the second domain of reverse transcriptase which has RNase H activity. The oligonucleotides
resulting from the digested virion RNA are then used as primers for the synthesis of plus sense cDNA using the newly made minus sense cDNA as a template. The process of reverse transcription is error-prone and contributes to genetic recombination and the high mutation rate of HIV-1 (1 in $10^6$) which are the major causes of HIV-1 diversity (Mansky LM, 1995). The linear double stranded DNA formed, together with Gag matrix (MA) protein, Vpr and the viral integrase forms the pre-integrase complex (Heinzyinger NK, 1994). The complex is transported to the nucleus through independent nuclear targeting mechanisms of DNA and Vpr proteins. Once in the nucleus, the integrase trims the linear double stranded DNA, cleaves the host’s chromosomal DNA and ligates together the free viral and host chromosome DNA (Katz RA, 1994).

The integrated viral DNA, or provirus, stays in latent state until activated. Tat, a viral transcriptional trans-activator protein, can activate the provirus by binding to the viral RNA element TAR, initiating the expression of proviral genes (Gaynor RB, 1995). The transcription is initiated at the 5’LTR and terminated at the 3’LTR (Weiss RA, 1982). The transcribed RNA can either be sliced into smaller viral RNA transported to the cytoplasm to serve as mRNA for the synthesis of Gag-Pol precursor proteins, or may be transported to the plasma membrane as genomic RNA to be incorporated into the virus particle during the budding stage (Shwartz S, 1990). The HIV genes can be divided into the early genes and the late genes. The early gene transcripts, tat, rev, and nef, are short while the late gene transcripts gag, pol, env, vpr, vpu, are longer in length. The change from early to late gene transcripts is under the control of the rev protein (Pomerantz R J, 1997). The presence of rev is critical for the expression and maintenance of the late HIV-1 gene transcripts in the cytoplasm of the infected cell.
The transcription of the viral DNA also produces viral structural proteins. These proteins are produced as precursor proteins and therefore have to be processed before being incorporated as part of the new virion. The envelope glycoprotein, gp160 is a precursor polyprotein in the endoplasmic reticulum (ER) and later processed by the host’s pro-protein convertases to yield gp120 and gp41, which remain non-covalently associated (Colman PM, 2003, Decroly E, 1994, Earl PL, 1991). Once all the necessary components for viral assembly are ready the viral budding process can begin. Gag and Gag-Pol precursors, processed envelope protein, viral RNA and the Vpr protein migrate to the cell surface where budding takes place forming an immature non-infectious virus particle. The viral protease (Pr) completes the maturation process by proteolytic processing of Gag and Gag-Pol precursor proteins forming an infectious virus particle (Craven RC, 1991) (Figure 3).
Figure 3: The life-cycle of HIV. The figure above outlines the various viral replication steps from adsorption to host cell receptor to viral budding and maturation.

1.4.3 HIV transmission

Once the virions are produced as a result of viral replication, the newly formed viruses can either remain in the host or they can be transmitted to a susceptible individual through the many transmission routes discussed below.

HIV can be transmitted via several routes. However, sexual transmission is considered the most prominent mode of HIV transmission globally. Indeed, it is estimated that sexual transmission by both heterosexual sex and sex between men accounts for about 85% of the global HIV burden (Simon V, 2006). However, compared to other modes of HIV transmission, the efficiency of HIV transmission via the sexual route is very low. It is estimated that the probability of HIV transmission per sexual act of vaginal intercourse results into 3 infections per 10,000 contacts among discordant couples (Royce RA, 1997) compared to 24 per 1000 exposures via blood transfusion (Baggaley RF, 2006). Although the efficiency for sexual transmission of HIV is low, it can also vary depending on other factors such as use of condom, presence of other sexually transmitted infection in the recipient person and the viral load in the transmitter. High viral load during acute HIV infection or advancing HIV disease (Wawer MJ, 2005), and/or co-infections with other STIs (Rottingen J, 2001), particularly those that cause genital ulcers, significantly increase the risk or the efficiency of HIV transmission. A study done in Uganda on discordant couples, found a higher probability (0.0041) of transmission per act among couples who reported genital ulcer disease compared to 0.0011 for couples who did not. In the same study, it was reported that HIV-1 transmission probability increased from 0.0001 per act at viral loads of less than 1700 copies/ml to 0.0023 per act at 38,500 copies/ml (Gray RH, 2001).
In addition to the risks outlined above, the risk of transmission through sexual route also varies depending on sexual practices. It is generally agreed that the risk of male-to-female transmission of HIV is higher than the risk of female-to-male transmission (Padian NS, 1991). However, some reports on this subject found out that this may not be true. One such a study found that in North America, rates of HIV transmission is twice as high in male-to-female transmission (23%) than for female-to-male (11%) (Mastro TM, 1996). On the other hand, two other studies found that in developing countries, rates of HIV transmission were higher in female-to-male (73%) transmission than in male-to-female (60%) transmission (Hira SK, 1990, O'Farrell N, 2001). Men in developing countries, unlike men in developed countries, were more likely to visit female commercial sex workers, thereby increasing their risk to HIV infection. Among homosexual men, receptive anal sex has been associated with greater risk of HIV transmission (DeGruttola V, 1989). HIV is, therefore, most likely transmitted via sexual intercourse as a result of multiple repeated exposures over time.

 Mothers who are HIV infected can also transmit the virus to their babies in utero, during the process of childbirth and through breast-feeding. The majority of these infections occur in the birth canal during childbirth. In the absence of treatment, around 15-30% of babies born to HIV positive women will become infected with HIV during pregnancy and delivery and a further 5-20% will become infected through breastfeeding (De Cock KM, 2000). While this route played a significant role in spread of HIV in children, administration of ARVs at birth to both the mother and the baby has greatly reduced transmission of HIV in this group.
Another route of transmission of HIV from host to host is via contaminated blood and blood products. Blood transfusion, like mother to child transmission played a major role in the spread of HIV at the beginning of the HIV/AIDS pandemic but has since become insignificant due to improvement in the safety of national blood supplies. Nearly all countries currently routinely screen blood for HIV antibodies and as such provide safe blood for transfusion. Contaminated injection equipment is still a major route of HIV transmission especially among intravenous drug users (IDU) in developed countries. Efforts made to provide clean needles and syringes have proven effective in reducing the risk of HIV transmission among IDU, without contributing to an increase in drug use (Hilton BA, 2001). However, these efforts are still a long way from achieving full effectiveness as drug use is illegal in many countries. Other problems such as stigmatization are also slowing down these efforts. By having clean needle services in specific locations, the fear of being identified with such facilities may prevent some IDUs from accessing such facilities. Overall, HIV can be transmitted via any of the routes outlined above. Once infected, the individual remains infected for life and without treatment, the ultimate outcome is death. The HIV disease course and pathogenesis that culminates in death has been well studied.

1.5 HIV disease course and pathogenesis

Unlike most infectious pathogens in which a single episode of symptoms is the culmination of the clinical disease, infection with HIV normally leads to three sequential clinical stages namely, acute, chronic infection and AIDS. The duration of this entire clinical presentation varies between individuals, with some people developing AIDS defining conditions after 2 years of infection while others can take up to 10 years before developing full blown AIDS (Bacchetti P, 1989).
1.5.1 Acute stage

The acute stage of HIV infection, which lasts for about 6-12 weeks from the time of infection to the time antibodies develop, is characterized by some patients presenting with 'typical' mononucleosis-like symptoms (Vanhems P, 1997). However, not all patients with acute HIV infection develop these symptoms. Indeed, it is estimated that only about 87% of these patients develop recognizable acute symptoms (Schacker T, 1996). Because these symptoms are non-specific, patients can present with fever, pharyngitis, fatigue, lymphadenopathy, weight loss, and myalgia (Cooper DA, 1985, Schacker T, 1996). The symptoms present within days or weeks after primary HIV infection and may last from a few days up to 10 weeks. Of these symptoms, fever, which is reported in the majority of seroconverting subjects (Vanhems P, 1999, Lavreys L, 2000), is the most common symptom. During this time the patient plasma viral load increases with the peak viraemia occurring within 6 to 15 days after the onset of symptoms (Clark SJ, 1991) at which time the donor is highly infectious (Quinn TC, 2000). Within weeks, the plasma viral load begins to decline reaching a viral 'set-point' at around 12–18 months after infection (Mellors JW, 1995). The decline in plasma viraemia seems to be due to host cellular immune responses but not due to HIV specific neutralizing antibodies which are rarely detected at this stage (Ariyoshi K, 1992). The rise in number of CD8+T cells and the detection of virus-specific cytotoxic CD8+ T lymphocytes within days after infection suggests the involvement of these cells in the clearance of virus in the peripheral blood (Koup RA, 1994). As the plasma viral load increases during primary HIV infection, the number of CD4 +T cells also declines (Figure 4). Detectable amounts of HIV specific antibodies appear three to six months after HIV infection and this coincides with symptom resolution, low viral load and low CD4+T counts in peripheral blood.
1.5.2 Chronic stage

The appearance of detectable HIV antibodies, or seroconversion, is followed by a long and variable asymptomatic HIV chronic or latent infection period before the development of AIDS. Chronic HIV clinical stage is characterized by low plasma viral load and slow decline in CD4+T cells over an average of approximately 10 years (Fauci AS, 1991, Lifson AR, 1991). Individuals who go through this disease course are referred to as Typical Progressors (TP). However, due to variability in time from HIV infection to development of AIDS, three other groups have been identified, namely; Rapid Progressors (RP) (Phair JP, 1994), Long-term Non-Progressors (LTNPs) (Cao Y, 1995) and Long term survivors (Schrager LJ, 1994). Rapid progressors constitute about 10–15% of HIV-infected individuals. They experience a rapid progression to AIDS within two to three years of primary infection (Phair JP, 1994). The suggested mechanism behind the rapid progression is that their immune system is unable to control the initial burst of viremia and as such the high viral burden is able to destroy the immune system thereby exposing the person to opportunistic infections within the first or second year after primary infection. The long-term non-progressors, unlike rapid progressors, form a small percentage (less than 5%) of HIV infected individuals. They do not experience progression of disease (Cao Y, 1995). They have CD4+ T-cell counts greater than 500 cells/ml (Schrager LJ, 1994, Sheppard HW, 1993). On the other hand, long-term survivors who consist of less than 5% of HIV-1 positive subjects can remain clinically healthy and immunologically normal for more than a decade (Schrager LJ, 1994).
1.5.3 AIDS Stage

After years of chronic HIV infection, the virus eventually destroys the ability of the host immune system to fight opportunistic infections (Table 1). At this stage, the patient is profoundly immunosuppressed, HIV-specific cytotoxic activity is generally lost, neutralizing antibodies are rarely detected, and the titers of antibodies against a variety of HIV proteins are significantly decreased (Haynes BF, 1996). This is followed by the appearance of one or a few opportunistic infections. Clinically, this stage of HIV infection is referred to as AIDS. Due to differences in clinical presentation of AIDS patients, both the World Health Organization (WHO) and CDC came-up with a universally accepted criteria for defining AIDS clinical condition (Wkly Epidemiol Rec, 1990). For example, according to the Centre for Disease Control and prevention (CDC) definition, one has AIDS if he/she is infected with HIV, has a CD4+ T-cell count below 200 cells/µl or a CD4+ T-cell percentage of total lymphocytes of less than 14% or has one of the AIDS defining illnesses listed in table 1 (MMWR 1987;36:1-15S).
Figure 4: Kinetic of HIV disease. The figure above shows various stages of HIV infection namely; acute, asymptomatic and AIDS. It also shows trends in immunological responses at any of the stages. Permission to use this graph was obtained from Dr. Keith Fowke, based on Alimonti J, et al, 2003.
Table 1: CDC classification of AIDS defining conditions

**Category C AIDS-Indicator Conditions**

1. Bacterial pneumonia, recurrent (≥2 episodes in 12 months)
2. Candidiasis of the bronchi, trachea, or lungs
3. Candidiasis, esophageal
4. Cervical carcinoma, invasive, confirmed by biopsy
5. Coccidioidomycosis, disseminated or extrapulmonary
6. Cryptococcosis, extrapulmonary
7. Cryptosporidiosis, chronic intestinal (>1-month duration)
8. Cytomegalovirus disease (other than liver, spleen, or nodes)
9. Encephalopathy, HIV-related
10. Herpes simplex: chronic ulcers (>1-month duration), or bronchitis, pneumonitis, or esophagitis
11. Histoplasmosis, disseminated or extrapulmonary
12. Isosporiasis, chronic intestinal (>1-month duration)
13. Kaposi sarcoma
14. Lymphoma, Burkitt, immunoblastic, or primary central nervous system
15. *Mycobacterium avium* complex (MAC) or *M. kansasii*, disseminated or extrapulmonary
16. *Mycobacterium tuberculosis*, pulmonary or extrapulmonary
17. *Mycobacterium*, other species or unidentified species, disseminated or extrapulmonary
18. *Pneumocystis jiroveci* (formerly *carinii*) pneumonia (PCP)
19. Progressive multifocal leukoencephalopathy (PML)
20. *Salmonella* septicemia, recurrent (nontyphoid)
21. Toxoplasmosis of brain
22. Wasting syndrome due to HIV (involuntary weight loss >10% of baseline body weight) associated with either chronic diarrhea (≥2 loose stools per day ≥1 month) or chronic weakness and documented fever ≥1 month
1.6 HIV pathogenesis

Infection with HIV in untreated persons leads to a gradual loss of peripheral CD4+ T cells. Given that CD4+ T ‘helper’ cells are crucial in coordinating cellular and humoral immune responses against exogenous antigens, the gradual loss or depletion of these sub-sets of T lymphocytes leads to immunodeficiency or Acquired Immunodeficiency Syndrome (AIDS) and ultimately death. The loss of CD4+T cells in HIV-1 infected individuals occurs in peripheral blood and in the mucosal tissues. While previous studies on the mechanisms of CD4+T cell depletion were focused on the activities in the peripheral blood, recent reports on HIV-1 and SIV studies have revealed that mucosal tissues, primarily the gastrointestinal tract, are major sites for early viral replication and CD4+ T-cell destruction (Veazey R, 2003). These reports have also found that the main reason for rapid loss of CD4+T cells in the mucosa is because the majority of mucosal CD4+ T-cells are activated, thereby providing a large number of target cells for HIV-1 infection. As well the majority of these cells express high levels of CCR5, a chemokine receptor required for HIV-1 entry (Poles MA, 2001). Additionally, alpha 4 beta 7(α4β7), a gut homing molecule that is expressed on memory CD4+T cells, has recently been shown to function as a receptor for HIV-1 (Arthos J, 2008). By having more than one receptor for HIV, the memory CD4+T cells in the gut provide more targets for HIV, making them easy to infect. Despite these new discoveries, it is generally agreed that HIV targets and kills CD4+T cells through a number of ways (see discussion). However, before one becomes infected with HIV and dies, one has to be exposed to the virus. It is also known that not everyone who is exposed to HIV would become infected, and even among the infected not everyone develops AIDS uniformly.
1.7 Susceptibility to HIV-1 infection and disease progression to AIDS

In HIV infection, there is variability between individuals with respect to susceptibility to infection as well as with time to progression to AIDS. Factors such as the nature of the infecting virus, host genetics and environment have been suggested to determine susceptibility to HIV infection and disease progression.

1.7.1 Viral Factors

Several viral factors can determine individuals’ susceptibility to infection as well as the rate of disease progression. Such factors include the subtype of HIV being transmitted, whether the transmitted virus is attenuated, the ability of the virus to undergo mutations to escape the host immune response or the ability of the virus to undergo viral tropism change or phenotypic switch.

(i) HIV subtypes.

HIV-1 has been classified into groups M (major), O (outlier) and N (non-M, non-O) based on phylogenetic analysis of HIV samples obtained from various parts of the globe. Group M which comprises nine subtypes designated by the letters A–D, F–H, J and K, constitutes the majority of HIV infection globally (Robertson DL, 2000). Subtype B is the most common subtype in the United States and Western Europe, whereas subtypes A, C, D, and E predominate in the developing world (Essex M, 1999).

HIV subtype B viruses, which are mainly associated with transmission of HIV among injection drug users (IDU), have been found by some studies to have less potential for heterosexual
transmission in comparison to subtype E viruses. According to these studies, subtype E, unlike subtype B has enhanced rates of infection in Langerhans cells due to its high tropism for these cells (Soto-Ramirez LE, 1996). Langerhan cells, which are found mainly in the cervix, are considered a possible HIV target and have been shown by some studies to be less susceptible to infection with subtype B viruses but more susceptible to infection with subtype E viruses (Soto-Ramirez LE, 1996). Based on these studies it is plausible to speculate that the risk of heterosexual transmission of HIV-1 depends on the circulating subtypes of HIV-1 in the population. However, some studies have found no differences in susceptibility to HIV-1 infections between subtypes B and E (Montpetit M, 1996). Indeed, one study compared the risk of transmission between subtypes B and E in women in the USA and Thailand (Cohn M, 2001). The only differences this study found was a higher degree of inflammation in the vaginal mucosa of HIV-1 positive women in a population where subtype E is predominant. The findings of this study suggested that the enhancement of HIV-1 transmission reported in previous studies may have been due to other factors. Due to the controversies in these studies, it is still unclear whether the subtypes of HIV-1 circulating in an area are the real factors enhancing susceptibility to HIV-1 infection or whether other factors also play a role. Further investigations are warranted to resolve these controversies.

In addition to their role in determining susceptibility to infection, the subtypes of HIV can also play a role in HIV disease progression. However, there are conflicting reports with respect to the role played by different HIV sub-types and HIV disease progression to AIDS. For example, a number of studies comparing infection of HIV subtype D and A and the rate of disease progression to AIDS found that infection with HIV subtype D, and not subtype A, was more
strongly associated with rapid progression to AIDS (Kaleebu P, 2002, Baeten JM, 2007). Other studies have not found such differences (Alaeus A, 1999). While some HIV-1 subtypes may have real association with disease progression to AIDS, the variations reported in the above studies could be caused by factors such as host genetic factors or environmental factors, as well as differences in study design and sample size.

(ii) Viral Escape

During all stages of HIV infection, the host mounts strong cellular and humoral immune responses to clear the invading virus. HIV has adopted a number of ways by which avoid such host immune responses. By undergoing mutation, HIV evolves into mutants that cannot be recognized by the HIV-specific neutralizing antibodies nor HIV-specific cytotoxic T lymphocytes. These escape mutants have been well documented in humans and in animal models (Allen TM, 2000, Barouch DH, 2002). The best example of the role played by viral immune escape and disease progression comes from studies of individuals with the HLA-B27 allele (Kelleher AD, 2001). Infection of HIV in these individuals results in a slow disease progression. However, the emergence of escape mutants late during the course of HIV infection has been shown to coincide with increased viral replication and progression to AIDS (Kelleher AD, 2001). While associations of escape mutants with HIV disease progression at individual levels have been well studied and documented, such associations have not been shown at population levels. The lack of such association at the population level may be due to other factors such as viral attenuation.
(iii) *Viral Attenuation*

Mutations in the HIV genome, in addition to resulting in escape mutants, can also result in attenuated viruses. Such mutations or deletions have been identified in the *nef, rev, vif, vpr, vpu* genes and result in viruses which have reduced ability to replicate in their host. Such inability to replicate well in the host can result in lower plasma viral loads and delayed disease progression (Greenough TC, 1994, Carl S, 2000). The most studied attenuated forms of HIV are those that occur in the *nef* gene. First reported among blood recipients infected with a *nef* defective HIV-1 from a single blood donor in a Sydney Blood Bank (Learmont J, 1992), infection with HIV-1 defective viruses were thought to lead to non-progression to AIDS. However, follow-up studies on these patients revealed an association with slow progression to disease (Learmont JC, 1999). Due to the low viral load and normal CD4 counts found in these patients, the majority of these individuals are now classified as LTNP (Learmont JC, 1999).

(iv) *Viral tropism or phenotypic switch*

Changes in HIV-1 *env* that affect viral tropism have been suggested to be important for progression to disease in HIV-1 infected individuals. The phenotypic switch from R5 virus to X4 virus during the asymptomatic stage of HIV infection has long been associated with faster disease progression (Berger E, 1998), although the mechanism of this effect is unclear (Berkowitz RD, 1998). The appearance of X4 HIV viruses during the asymptomatic period of HIV infection has been associated with accelerated decline in CD4-positive lymphocyte counts and faster progression to disease. However, the fact that about 50% of individuals infected with R5 HIV virus still progress to AIDS raises the issue of whether other factors such as host factors are involved in the pathogenesis of AIDS.
1.7.2 Host Factors

While viral factors that affect host susceptibility to HIV infection and disease progression are well documented, it is evident that there are other factors that affect susceptibility. Indeed, other factors such as host genetics have been described as well. Host genetics can further be divided into two major groups namely; factors that modulate HIV entry and factors that regulate immune responses.

(i) Genetic factors that regulate immune responses

The human leukocyte antigen (HLA) class I and class II proteins play a fundamental role in regulating adaptive immune responses and as such are classified as the host factors that regulate the immune response. Therefore, possession of certain HLA allele may determine an individuals' ability or inability to mount a strong immune response to the invading pathogen. Such responses can in turn determine individual susceptibility to infection or the rate at which the disease progresses. The HLA class I molecules bind peptides derived from intracellular pathogens and present them to CD8+T cells, thereby initiating a cytotoxic T cell response. On the other hand, HLA class II molecules present to CD4+T cells peptides of extracellular origin. The differential abilities of any of these molecules to present peptides to either CD4+T cells or CD8+T cells can influence the outcome of infection or disease. As a consequence, studies done on HLA associations with infectious diseases have found a number of convincing HLA alleles associated with infectious diseases (Cooke GS, 2001). For example, a protective effect of HLA-B*53 against malaria has been observed in West Africa (Hill AV, 1991) while HLA-DR2 antigens have been shown to increase susceptibility to mycobacterial diseases (Visentainer JE, 1997). The role played by HLA antigens play in determining susceptibility to HIV infection and disease.
progression is well documented. For example, having identical HLA class I alleles between potential virus donors and recipients has been identified as a risk to increased susceptibility to HIV infection of the latter (MacDonald KS, 1998, Polycarpou A, 2002). Additionally, studies of HLA alleles and disease association have found that HLA-B*27 and B*57 (Kaslow RA, 1996, Migueles SA, 2000) correlate with slower HIV-1 disease progression while B*35 (Gao X, 2001, Scorza Smeraldi R, 1986) is associated with rapid AIDS onset.

The lack of diversity at class I loci has been found to be strongly associated with poor control of and relatively rapid disease progression (Carrington M, 1999). Although the exact mechanism of why the lack of diversity of HLA class I allele is associated with rapid disease progression is unknown, having heterozygous HLA loci is associated with ability to present a greater range of antigenic peptides to CTLs, resulting in a more protective immune response (Carrington M, 1999). The examples described above suggest that the variations in susceptibility to infection and disease progression can be partially determined by the genetically determined immune responses to HIV.

(ii) Genetic factors that modulate HIV entry

To initiate infection, the HIV-1 envelope glycoprotein, gp120, sequentially interacts with cellular receptors, CD4 and a chemokine co-receptor. Although there are many chemokines receptors, to date, CCR5 and CXCR4 are considered are to be the necessary co-receptors to R5 and X4 HIV viruses respectively (Littman DR, 1998). The discovery that CD4, chemokine receptors and their ligands can modulate the efficiency of HIV infection has expanded the scope of host factors that may impact on the susceptibility to HIV infection and the pathogenesis of HIV disease. The
alteration of these genetic factors either at the levels of expression or in functional differences of protein variants may lead to differences in permissiveness to HIV-1 infection and regulate the degree to which the virus can replicate in the infected host. The alteration of these proteins can be brought about by mechanisms such as genetic polymorphisms in the genes that code for these proteins. While the CD4 glycoprotein has been considered to be highly conserved, several polymorphisms have been detected in the genes that code for the chemokine receptor proteins and their ligands. Mutations in the CCR5, CCR2, CX(3)CR1, CXCL12 (SDF1), and CCL5 (RANTES) genes have been identified and associated with host resistance and/or susceptibility to HIV-1 infection and disease progression (Reiche EM, 2007). However, the best characterized of these genes is the CCR5-Δ32 mutation in the CCR5 gene. The 32-bp deletion within the coding region of the CCR5 gene introduces a premature stop codon, resulting in a defective molecule that is not transported to the cellular surface (Liu R, 1996, Samson M, 1996). Individuals homozygous for this gene mutation are resistant to HIV infection, whereas those having heterozygous alleles have been associated with a significant delay in disease progression (Dean M, 1996). Several other polymorphisms in the chemokine genes as well as in the CD4 gene that may affect host susceptibility to HIV infection and disease progression have been identified and will be discussed later.

1.8.0. Single nucleotide polymorphisms and HIV/AIDS

Single nucleotide polymorphisms (SNPs), substitutions of a novel nucleotide for a wild-type nucleotide within genomic DNA, are the most common type of genetic variations and account for about 90% of all genetic variation in humans (Collins FC, 1997). They are abundant, relatively stable evolutionarily, and distributed throughout the human genome. Many SNPs are believed to cause phenotypic differences and can be related to an individual’s susceptibility to disease. SNPs in
coding regions that cause amino acid changes, or the non-synonymous SNPs (nsSNPs) are believed to have the highest impact on phenotype as they directly affect protein structure and function (Collins FC, 1997). The altered protein structure could result in protein mis-folding or altered protein expression and if the protein is a receptor, its normal function may be modified by several mechanisms, including disruption of receptor-ligand binding and scaffolding accessory proteins (Wenkert D, 1996). SNPs may also interrupt the initiation or the termination of a codon, all with consequences for insufficient or prematurely truncated peptides. Overall, the human genetic variants, or changes caused by these SNPs, can influence the outcome of exposure to an infectious agent.

In HIV infection, SNPs have been found to be important in determining whether individuals exposed to HIV-1 become infected and if they do how quickly they develop AIDS. Several SNPs have been identified in the genes including those of the HIV-1 co-receptors, their ligands, cytokines and their receptors, transcription factors and immune response genes (O'Brien SJ., 2000). The influence of these SNPs on regulating the outcome of exposure to HIV and the subsequent impact on modifying the rate of disease progression is explored in the discussion section. However, while more focus has been put on the impact of SNPs on the HIV-1 co-receptors, no information is currently available in the literature on the effect SNPs in the HIV-1 receptor, CD4 molecule, could have on susceptibility to HIV-1 infection. The following sections will highlight what is currently known about CD4 polymorphism and the possible roles it may play in both susceptibility to HIV-1 infection and disease progression.
1.9.0 CD4 membrane glycoprotein

1.9.1 Structure and functions

CD4 antigen was one of the first lymphocyte cell surface antigens to be discovered. First discovered in mice in 1977 (Williams AF, 1977), the human CD4 was discovered two years later by use of the monoclonal antibody (mAb) OKT4 (Reinherz EL, 1979). CD4, a 55-kD glycoprotein, is predominantly expressed on the cell surface of helper T lymphocyte which are major histocompatibility class II restricted (Maddon PJ, 1985). CD4 is also expressed at low levels on the cells of monocyte/macrophage lineage, dendritic cells, granulocytes and eosinophils. The CD4 gene is located on the short arm of human chromosome 12, at region p12-pter (Isobe M, 1986). Subsequent studies on the amino acid sequence of CD4 indicated that it is divided into three segments namely; the extracellular segment that consist of four domains (D1-D4, residues 1-371), the trans-membrane segment (residues 372-395), and the intracellular segment or the cytoplasmic tail (residues 396-433) (Maddon PJ, 1987, Clark SJ, 1987) (Figure 5). Of note are the important epitopes on both the extracellular segment and the cytoplasmic tail of CD4. The extracellular domains contain the following binding sites; D1 for gp120 of HIV, the extended region of D1 and D2 for MHC II of antigen presenting cells, D3 for TCR of T lymphocytes (Vignali D, 1999), D4 for IL-16(Liu Y, 1999) and Human Herpes Virus 7 binds to D1-D4 (Lusso P, 1994). Unlike the extracellular segment of CD4 that has many binding sites, the intracellular or the cytoplasmic tail of CD4 has three important binding sites for T-lymphocyte lck, HIV vpu and HIV-1 nef (Willey RL, 1994, Preusser A, 2001).
The primary function of CD4 is that of a co-receptor with the T cell antigen receptor (TCR). By binding to the non-polymorphic region on MHC II of antigen presenting cells, CD4 is able to augment the signaling activity of the TCR thereby enhancing the T cell response. CD4 functions as a signaling receptor and this function is dependant on its association with p56\textsuperscript{ck}, a src-family tyrosine kinase. By binding to MHC II on an antigen presenting cell, CD4 transduces signals through p56\textsuperscript{ck} which becomes phosphorylated and activated.
Figure 5: Structure of Human CD4: CD4 molecule consists of Extra-Cellular Domains, D1-D4 (Amino-Acid sequence 1-371), Trans-Membrane(TM)-(Amino Acid sequence 372-395) and a cytoplasmic tail (Amino Acid sequences 396-433).
1.9.2 The function of CD4 as a signal transduction receptor

CD4 can function as a signaling molecule. Studies have demonstrated that cross-linking CD4 and TCR/CD3 with monoclonal antibodies caused significantly higher T cell activation than if the TCR/CD3 complex were cross-linked alone (Anderson P, 1987). On the other hand, cross-linking CD4 alone has been shown to inhibit T lymphocyte activation (Anderson P, 1987, Walker C, 1987). These studies suggest that the enhancement of T lymphocyte responsiveness due to CD4 signaling is only possible if both CD4 and TCR/CD3 are engaged in the signaling process. Although the extracellular segment of CD4 participates in the interaction between CD4 and MHC II of antigen presenting cell to initiate the signal, it is the cytoplasmic region of CD4 that has been found to be essential for this function. Indeed, p56\(^{lck}\) which is non-covalently linked to the cytoplasmic tail of CD4 has been suggested, at least in part, to regulate the signaling function of CD4. Evidence of such involvement came from studies in which cross-linking of CD4 with monoclonal antibodies resulted in a rapid increase in the phosphorylation of p56\(^{lck}\) on tyrosine residues (Luo K, 1990).

In general, the binding of CD4 to either MHC-II of an antigen presenting cell or to an antigen, transduces signals that are passed over to p56\(^{lck}\) which becomes phosphorylated. Upon phosphorylation, lck either becomes activated or inactivated. The activation state of p56\(^{lck}\) is dependent on whether more negative regulator (tyr505) or positive regulator (tyr394) is phosphorylated (figure 6b). Phosphorylated lck moves to the TCR complex where it associates and phosphorylates ZAP-70 (zeta chain associated protein of 70 kDa). ZAP-70 then phosphorylates LAT (linker for activation protein). LAT also attracts two phosphotyrosine-binding proteins namely; SOS (son of seven less) and isoform of phospho lipase C (PL\(\gamma\)1).
Each of these proteins then initiates a separate signaling pathway, namely the Ras/MAPK pathway and phosphatidylinositol pathways. The Ras/MAPK pathway culminates in phosphorylation of transcription factor ERK, which has the ability to transcribe genes that promote IL-2 activity. On the other hand, the phosphatidylinositol pathway culminates in the activation of another transcription factor, NF-kB, a family of transcription factors that is involved in regulation of many genes including cytokines (Figure 6a).
Figure 6a: The proposed CD4-specific signaling pathway. The binding of CD4 to antigen or MHC-II of antigen presenting cells transduces a signal that induces phosphorylation of p56lck. This initiates a cascade of events that eventually results in translocation of NF-kβ to the nucleus.
Figure 6b: Regulation of p56lck enzymatic activity: The diagram above shows the postulated conformation of active and inactive lck. Phosphorylation of tyr505 leads to an inactive conformation of lck. Dephosphorylation of tyr505, which is controlled by the protein tyrosine phosphatase CD45, results in autophosphorylation of tyr394 which in turn results in activation of lck.
1.9.3 The role of CD4 as a receptor to HIV

The discovery that HIV specifically infects, CD4+T cells led to the investigation of the role played by CD4 in mediating the HIV entry process. Evidence that CD4 antigen is the receptor for HIV came from in vitro studies where anti-CD4 antibody inhibited HIV infection of CD4+ cells (Dalgleish AG, 1984). This role was further confirmed when expression of CD4 was shown to confer HIV susceptibility on previously non-susceptible cells (Maddon PJ, 1986). Although these studies suggested that CD4 was necessary for HIV entry into a host cell, it also became clear that presence of CD4 alone was neither sufficient nor always necessary for virus entry. Indeed, it was later discovered that for HIV to enter a host cell, it requires a receptor (CD4) and a chemokine co-receptor (CCR5 or CXCR4). Furthermore, in rare cases, HIV was found to infect CD4-negative cells. Infection of CD4-negative cells is rare with HIV-1, however, certain strains of HIV-2 have been found to infect some human cell lines independent of CD4 (Clapham P, 1992). The clinical relevance of CD4-independent infection is not clear.

1.9.4 The role of CD4-gp120 interaction in HIV-1 immunodeficiency.

In addition to its role as a receptor to HIV, there is compelling evidence to suggest that the high binding affinity between CD4 on T lymphocytes and gp120 plays an important role in several complex pathological mechanisms in HIV infection and may partially explain the mechanism of CD4 decline. First, the high binding affinity between gp120 and CD4 may initiate syncytia formation and subsequently lead to syncytium-induced cell death (Sodroski J, 1986). Second, soluble gp120 can bind to CD4 on non-infected CD4+T cells and kill them by antibody dependant cell-mediated cytotoxicity (Hammond SA, 1992). Additionally, the binding of shed gp120 to CD4 molecules may initiate stronger signals that lead to phosphorylation of
intracellular proteins such as p56<sup>ck</sup> and ZAP70. The phosphorylation of these proteins results in a cascade of events leading to programmed cell death (Banda NK, 1992). Because the interaction of CD4 and gp120 may lead to several pathological mechanisms, genetic alteration in the CD4 gene could greatly affect these processes.

1.10 CD4 polymorphism

Although CD4 had been assumed by many researchers to be non-polymorphic, Bach et al first reported the presence of a rare non-synonymous single nucleotide polymorphism (SNP) on human inducer T cells in 1981 (Bach MA, 1981). Sequence analysis of the third(D3) and fourth(D4) domains of CD4 from individuals whose CD4 were non-reactive to anti-OKT4 revealed a sequence that is identical to normal CD4 except for a transition of cytosine to thymidine at nucleotide position 868(C868T) (Hodge TW, 1991). Amino acid sequence of CD4 from peripheral blood mononuclear cells (PBMCs) from individuals lacking reactivity with anti-OKT4 demonstrated that the inability of anti-OKT4 to bind its epitope on CD4 was due to a single amino acid substitution of tryptophan for arginine at amino acid residue 240 in the D3 extracellular domain of the CD4 molecule (Lederman S, 1991, Hodge TW, 1991). The resulting isoform of CD4 has been named as CD4-Trp240(868T) based on the location of the amino acid on CD4 genome. The replacement of the basic arginine residue with the more hydrophobic tryptophan in the centre of a β sheet was predicted to have a significant effect on the tertiary structure (Maddon PJ, 1987). These and our own studies (see discussion) lead us to hypothesize a role of the C868T polymorphism in altered susceptibility to HIV infection and investigate the molecular mechanisms.
2.0 Hypotheses and specific objectives.

2.1 Study rationale:

Since the discovery of HIV-1 in 1981, the cumulative total of individuals infected with HIV-1 and deaths due to AIDS exceeds 60 million and 25 million people, respectively (UNAIDS, 2007). The majority of these infections are found in Sub-Saharan Africa. It is now known that not all people exposed to HIV-1 become infected, and those who do progress to AIDS-defining pathology do so at different time intervals. The inter-individual variability observed in terms of acquisition of infection and disease progression is governed by many factors including host genetic factors. Among the most studied host genetic factors that regulate susceptibility to HIV-1 infection are the genetic variants in the chemokine receptors and their ligands. A recently discovered genetic variant in CD4 gene, C868T has not been investigated for its possible role in HIV infection and disease progression.

However, epidemiological studies done by our group have shed some light on the possible role of this genetic variant in HIV infection as well as disease progression. A master’s student in our laboratory, Françoise Vouriot who performed this investigation, made the following observations; (i): that CD4 868T is associated with increased susceptibility to HIV infection (figure 7) and (ii); upon infection these subjects progress to AIDS faster compared to those with wild-type CD4 (figure 8). Although these studies were a useful first step, one of the fundamental questions they left unanswered was the molecular mechanism behind the association. It is due to this gap in knowledge that we seek to investigate the molecular basis of CD4 868T in enhancing susceptibility to HIV infection as well as disease progression.
CD4 868T Polymorphism and HIV-1 Seroconversion

Figure 7: Kaplan-Meier plot of time from enrollment to HIV-1 seroconversion. Forty-eight individuals with known date of HIV-1 seroconversion were included in this analysis: 19 had CD4 C868T (*), 29 were homozygous for the wild type allele CD4 868CC (○). Log rank test p = 0.0046 and Wilcoxon test p = 0.0088. Data points at drops in the line represent a clinical event (seroconversion) while icons between drops represent the end of an individual’s data set (censoring event). Used with permission from Françoise Vouriot (MSc thesis, 2005).
Figure 8: Kaplan Meier plot of MCH CD4+ T-cell decline to less than 400 cells/cubic mm. Of the 159 seropositive individuals used for this analysis, 113 were homozygous for the wild type allele (diamonds), 40 were heterozygous (asterisks) and 6 were homozygous for 868T (triangles). Log rank test $p = 0.04$. Used with permission from Françoise Vouriot (MSc Thesis, 2005).
2.2 Hypotheses

Based on the epidemiological findings of C868T being associated with increased susceptibility to HIV-1 infection and a predicted change in CD4 structure, we hypothesize that:

1. Changes in the molecular structure of CD4 alter its affinity as a cellular receptor to gp120, thus allowing HIV-1 to infect more readily.

2. CD4 specific signal transduction is greatly enhanced in polymorphic CD4 molecule which results in increased susceptibility to HIV infection and apoptotic cell death.

2.3. Specific Objectives:

To test the above hypotheses the following specific objectives were proposed;

1. To investigate if CD4 868T cells are more susceptible to infection with HIV-1 virus than the wild type CD4.

2. To investigate the differences in binding affinities between gp120 and the two CD4 isoforms.

3. To determine if signal transduction is enhanced in the CD4 868T compared to wild-type CD4 and assess the consequences of the activation.
3.0. MATERIALS AND METHODS

3.1. Source of Biological Materials

*Source of the cell lines used in this study.*

A CD4 negative, human T-cell line A2.01 was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Original donation by Dr. Thomas Folks. This cell line was chosen for use in this project for three reasons. First, it is derived from CD4+ T cell parent (A3.01 cell line). Second, it does not express CD4 and thirdly, it expresses CXCR4 one of the chemokine co-receptors that HIV-1 uses to enter a host cell.

*CD4 hybridoma* (SIM.4)

SIM.4 hybridoma cells were grown and maintained at $10^5$ cells/ml as single cells in suspension. SIM.4 antibodies recognize human CD4 at the leu-3a epitope (D1 of CD4 domain). SIM.4 antibodies have been shown to block HIV-induced syncytium formation. These cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Original donation by Dr. James Hildreth.

*Source of Peripheral Blood Mononuclear cells (PBMCs)*

The subjects used in this study consisted of HIV negative healthy individuals of Caucasian origin and people of African decent from Winnipeg Manitoba. Blood samples were collected from the subjects according to University of Manitoba Ethics Review Board approved protocol.
Sources of Viruses used in this study.

HIV-1_{HIB} was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HTLV-III_{B}/H9 original donation by Dr. Robert Gallo. HIV-1_{BAL} was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: original donation by Dr. Suzanne Gartner, Dr. Mikulas Popovic and Dr. Robert Gallo. Kenyan primary viral isolates ml 235 and ml 1956 were derived from the Pumwani sex workers cohort. The viruses had been obtained by co-culturing of PHA stimulated PBMCs from HIV uninfected blood donors from Winnipeg, Manitoba with HIV infected PBMCs from commercial sex workers from the Pumwani cohort of commercial sex workers in Nairobi Kenya (Land, A.M, 2008). In brief, co-culture of patient and donor PBMC was carried out in a 24-well tissue culture flask at a ratio of 1:1 and incubated at 37°C in 5% CO₂ using humidified incubators. Fifty percent replacement of culture media was made three times a week and the culture supernatant removed was saved for virus detection assays. Cultures were supplemented with fresh PHA stimulated donor PBMC at weekly intervals for 4-weeks. The culture supernatant with the highest p24 antigen was used to propagate virus for infection experiments.

HIV-1 pseudotype virus

A VSV-G pseudotyped virus HIV-1 PNL 4.3 Luc+/Env was kindly donated by Dr. Xiaojian Yao. The following steps describe how the pseudovirus was generated. A plasmid pNL-Bru/Env-luc+ which has an HIV backbone but had Env gene knocked out and nef gene replaced by firefly indicator luciferase gene. Another plasmid SVCMV-VSV-G that encodes the vesicular stomatitis virus G protein was used to co-transfect 293T cells (Ao Z, 2007). In brief, plasmids prepared in 500µl of HeBS buffer were added to a Petri dish with a monolayer of 293T cells that had been
cultured overnight. The cell culture and plasmids were gently mixed and incubated at for 8 to 12 hours. The culture supernatant was removed and replaced with fresh RPMI 1640 containing 20% FSC. After 40 hours of further incubation, the culture supernatant were harvested, filtered using a 0.45μM syringe filter and concentrated using a Centricon column (Millipore, Catalogue number: 91008, USA). The viral titer of the concentrated virus was determined using a reverse transcriptase assay. In brief, 50μl of 1:10, 1:100, 1:200 diluted pseudovirus concentrate were added into reaction tubes containing Tris Hydrochloride, MgCl₂, KCl, EDTA, Triton X-100, Ethylene glycol stock solution and H₂O. Additional reagents such as DDT (dithiotheritol), GSH (reduced gluthatione), Poly-A-oligo (DT) and ³HTTP were added into each tube and incubated at 33°C for 22 hours. The reaction was stopped by adding 1 ml of refrigerated 10% trichloroacetic acid (TCA) and the virus harvested on a pre-wetted filter papers (Fisher Scientific, Catalogue number 09-804-24C). Finally, scintillation counting was done for each membrane resuspended in 5μl of scintillation liquid using a Beckman scintillation counter (Liquid scintillation System, Beckman, LS6000TA). A viral titer of 5cpm/cell was determined to be suitable for use in infection assays.
3.2 General Chemicals

Unless specified, all the chemicals used in this study were obtained from Sigma.

3.3 Culture Reagents

Reagent for A2.01 Cells

A2.01 cells were propagated in RPMI 1640 (Gibco) supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml) (Invitrogen), L-glutamine (300 μg/ml) (Gibco-BRL).

Reagents for A2.01/868T and A2.01/868C cells.

The transfected A2.01 cells were cultured in RPMI 1640 (Gibco) supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml) (Invitrogen), L-glutamine (300 μg/ml) (Gibco-BRL) and the antibiotics G418 (1 mg/ml) (Invitrogen).

Freezing Medium for A2.01 Cells

Freezing medium was prepared by combining 90% heat inactivated fetal calf serum (Invitrogen) and 10% DMSO (Sigma). One milliliter of freezing media was used to freeze down 1 x 10^7 cells.

Reagents for CD4 hybridoma (SIM.4)

The CD4 hybridoma cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine, 10 mM HEPES, 0.2 U/ml insulin, 0.05 mg/ml pyruvate, 0.15 mg/ml oxalacetic acid, and 5 x 10^{-5} M 80% β-mercaptoethanol, 10% fetal calf serum, 10% NCTC-135 media(Sigma, catalogue number N3262).
Reagents for purification of SIM.4 monoclonal antibody

Binding buffer (0.02 M Sodium Phosphate)

To make the binding buffer, 0.4M sodium phosphate dibasic (Sigma) was mixed with 0.4M sodium phosphate monobasic. The solution pH adjusted to pH 7.0, diluted 1:10 with dH2O and filtered ready for use.

Elution buffer (0.1M Glycine, pH 2.7)

The Elution buffer was made by dissolving glycine in 400ml of double distilled water and then pH adjusted to 2.7 using HCl before being filtered.

Tris-HCl buffer (1.0M, pH 9.0)

The buffer was made by dissolving TRIS-powder in 450ml of distilled water and adjusting the pH to 9.0 using HCl. The solution was filtered before use.

Reagent for DNA Isolation

QIAamp DNA Mini Kit (Qiagen, catalogue number 51304). It contains the following reagents:

- QIAGEN Protease (contains proteinase K enzyme) used for cell lysis.
- Buffer AL (lysis buffer)
- Buffer AW1 (wash buffer)
- Buffer AW2 (wash buffer)
- Buffer AE(Elution buffer)
- QIAamp Mini Spin Columns for purification and collection of sample DNA.

For a brief description of the protocol, see section 3.6.3 under general methods.
3.4 Reagents for specific methods

3.4.1 Reagents for signal transduction studies

(i) Western blot Assay

*Lysis buffer (1x)*

Lysis buffer consisted of 10mM Tris-HCl pH 7.5, 30mM Na pyrophosphate pH 7.6, 5mM EDTA, 150mM NaCl, 50mM NaF, 1% NP-40, 10μg/ml aprotinin, 10μg/ml leupeptin, 2μg/ml pepstatin A and 1mM NaVO₄.

*Goat Anti-Mouse (GAM)*

A solution of 0.135 M sodium chloride was prepared and 2ml added to a vial containing lyophilized GAM powder (Sigma, catalogue number M8769). The vial was gently rotated until all the powder was completely dissolved and a final concentration of 1 mg/ml solution achieved. The solution was aliquoted into 100μl volumes and frozen at -20°C.

*Separation solution: 10% SDS-PAGE Gel*

The gel was made up of 3.6 ml of double distilled water, 2.5ml of 1.5M Tris, 0.1ml of 10% SDS, 3.33ml of acrylamide, 0.005ml of Tetramethylenelediamine (TEMED), and 0.5ml of 1% ammonium (Am) per sulfate.

*Stacking SDS-PAGE Gel*

The gel was made up of 3.05ml of double distilled water, 1.25ml of 0.5M Tris, 0.05ml of 10% SDS, 0.65ml of Acrylamide, 0.005ml TEMED and 1% Ammonium sulfate.
Western blot wash buffer (10x)

The wash buffer was made-up of 10mM Tris-HCl pH 8.0, 150mM NaCl, and 0.05% Tween-20 in 500ml of dH2O.

Western blot blocking solution

The blocking solution consisted of 5% skim milk in wash buffer.

Western blot transfer buffer (10x)

Consisted of 30.22gm of Tris-HCl base and 144.13gm of Glycerol dissolved in 1litre of dH2O.

Western blot running buffer

Running buffer was made by adding 12gm of TRIS base, 57.6gm of Glycine and 4gm of SDS to 4 liters of dH2O.

Western blot loading buffer

loading buffer (4x) was made up of 25ml of 4x Tris HCl/SDS pH 6.8, 20ml of glycerol, 4gms of SDS, 2ml of 2-Mercaptoethanol l(ME) and 1gm Bromophenol Blue.

Western Blot detection kit

ECL Advance TM Western Blotting Detection Kit (Amersham Biosciences, Catalog number RPN2135). Equal volumes of Detection Reagent 1 and Detection reagent 2 were added in 5ml falcon tube and completely covered with Aluminum foil to protect the solution from light.
(ii) Flow Cytometry Assay

The following reagents were used for intracellular staining of HIV p24 antigen for flow cytometry analysis.

- BD perm/Wash Buffer 1 (BD Biosciences, catalog number 557885).
- BD flow fix buffer 1(BD Biosciences, catalog number 557870).

Wash buffer

Wash buffer was made by adding 1ml of FCS (Gibco) to 49ml of 1x Phosphate Buffered Saline (PBS) to make PBS with 2% FCS.

Cell fix solution or 10% Paraformaldehyde (PFA)

The cell fix solution was made by adding 5gm of PFA (Sigma) and 2ml of 5M NaCl to 48ml of ddH2O. The solution was heated for approximately 1 minute and 20 µl of 10M NaOH added to help in the solution dissolve completely. It is recommended to make this solution fresh on weekly basis.

(iii) Luminex Assay

The following reagents were used for luminex assay (See section 3.7.3).

Beadlyte Anti-Lck Beads (Upstate, catalog number 42-617).

Beadlyte Anti-Lck Biotin (Upstate, Catalog number 44-617)

Beadlyte cell signaling lysis buffer A (Upstate, Catalog number 43-018)

Beadlyte Cell signaling Buffer Kit (Upstate, Catalog number 48-600)
3.4.2 Reagents for HIV-1 infection studies.

(i) Reagents for propagation of HIV virus stocks in PBMC’s

PBMC’s were propagated in RPMI (Gibco) with 10% fetal calf serum (Hyclone), polybrene (2 ug/ml) (Sigma), penicillin G sodium (50 units/ml) and streptomycin sulfate (50 units/ml) (Gibco), and rIL-2 (10 units/ml) (NIH AIDS Reference and Reagents Program).

(ii) HIV-1 p24 ELISA

Coating antibody (NIH Reference and Reagent Program)

The HIV-1 p24 monoclonal antibody was diluted in coating buffer to a final concentration of 2µg/ml. One hundred microliters of the diluted antibody was added to all the wells of a 96-well plate and incubated at 4°C overnight. The unbound excess antibody was discarded and the plates stored at -70°C.

HIV-1 p24 Hybridoma (183-H12-5C)

The HIV-1 p24 hybridoma cells were cultured in RPMI-10% FCS for about two to three weeks. The antibody was purified from spent supernatant using Protein-A sepharose. Eluted antibody was dialyzed versus PBS and using an extinction coefficient of 1.2, the concentration was determined. Antibody was stored at -70°C.

Rabbit polyclonal anti-p24 mAb (NIH Reference and Reagent Program)

The Rabbit polyclonal anti-p24 was diluted 1:50 with PBS-T20-1% BSA (blocking buffer) and was stored at -70°C. Prior to use in assay, the antibody was diluted to 1:100 with glycerol,
which was stable at -20°C. It was then used in the p24 assay such that a final dilution of 1:20,000 was obtained (50μl of 1:100 in 10 ml of blocking buffer).

*Biotin anti-rabbit IgG (Sigma)*

The anti-rabbit IgG was diluted to 1:100 with equal volumes of blocking buffer (see below), and glycerol. It was stored at -20°C, and used at a final concentration of 1:4000 (or 10 μl in 10 ml of blocking buffer).

*Streptavidin-Alkaline-Phosphatase (SAAP).*

One milliliter of blocking buffer and two milliliters of glycerol were added to 1ml of SAAP (Jackson Immuno). The solution was then stored at -20°C until use. For use, 10 μl of the above solution was mixed with 10 ml of blocking buffer for a final concentration of 1:4000.

*104 phosphatase substrate (Sigma, catalogue number 104105)*

One tablet of phosphatase substrate was dissolved in 5 ml of 10% diethanolamine (DEA) (Sigma D-0681) buffer. Two tablets and 10 ml of DEA were required for a one 96-well ELISA plate. DEA buffer was made by combining 97 ml of DEA, and 100 mg MgCl₂, then increasing the volume to one litre. The pH was then adjusted as necessary to 9.8.

*P24 coupling Buffer*

Carbonate-bicarbonate (pH 9.6) was made by mixing 1.59 grams of Na₂CO₃ with 2.93 grams of Na₂HCO₃ per litre of water.
**P24 blocking Buffer**

Blocking buffer was made by adding 0.1% tween20 and 1% BSA to PBS.

**P24 wash Buffer**

Wash buffer was made by diluting 10X wash buffer (Amplicor) with distilled water (1 part wash buffer: 9 parts distilled water).

(iii) Luciferase assay

Luciferase Assay Kit (Promega, Catalog number E1500).

The kit contains the following reagents;

- Luciferase assay buffer (1×) (Catalogue number E153A).
- Luciferase assay substrate (lyophilized) (Catalogue number E151A).
- Luciferase cell culture lysis buffer (1×) (Catalogue number E152A).

Lyophilized Luciferase substrate was dissolved in 10ml of Luciferase buffer and aliquots of 1 ml frozen down in -20°C. For a brief description of the protocol, see section 3.7.2 under Luciferase assay.

### 3.4.3 Reagents for binding affinity studies-ELISA

**Coating Buffer** (0.15 M sodium carbonate and 0.35 M sodium bicarbonate)-pH 9.6.

The coating buffer was made by adding 3.18 g Na₂CO₃ and 5.86 g NaHCO₃ to 200 ml of H₂O and adjusting pH to 9.6 with HCl.
Wash solution

Wash buffer was made by adding 0.2 ml Tween-20 to 400 ml 1x PBS.

Blocking Solution (1x PBS with 1% BSA).

Blocking buffer was made by adding 5g of Bovine Serum Albumin (BSA) to 500ml of PBS.

3.5 Equipment used

1: Luminex 100 System (Luminex Corporation, Product number CN-L006-01) was used for reading plates for the Luminex assay.

2: EG and G Berthold Microplate Luminometer (Model LB 96V) was used for Luciferase assay.

3: MJ Research PTC-200 Thermal Cycler (MJ Research, Inc.) was used for amplification of DNA for CD4 SNP assays.

4: Well wash 384-(Thermo Labsystems) was used for washing ELISA plates.

5: SPECTRA-max plus (Molecular Devices) was used for reading ELISA assays.

6: Incubators (VWR).

7: Centrifuges (Allegra 6R Centrifuge, Beckman Coulter).

8: Gel Doc 2000(Bio-Rad Laboratories, Segrate, Italy) was used for Western blot assay.

9: Power Pack (Bio-Rad, Model number 300) was used for Western blot assay.

10: FASCalibur (BD Bioscinces) was used for immunophenotyping assays.

11: Trans-blot SD Semi-dry Transfer Cell (Bio-Rad) was used for Western blot assay.
3.6 General Methods

3.6.1 Cloning of wild-type and mutant CD4 genes

The wild-type and CD4 868T genes were originally cloned by Dr. Keith Fowke into pBluescript and DNA sequence confirmed. All sub-cloning was done using Invitrogen’s Gateway technology. The CD4 868C and CD4 868T genes were PCR amplified from the pBS CD4 clones using attaB containing primers. This allowed synthesis of CD4 products that were efficient substrates for recombination with the entry vector pDONOR201. The CD4 gene was then transferred from the pdonor201 into a mammalian expression vector pDEST 12.2 via the Ligase Reaction (LR) reaction. Clones were confirmed by DNA sequencing on an ABI3100. The clones were then amplified and used to transfect A2.01 cells.

3.6.2 Transfection of A2.01 cell line with CD4 868C and CD4 868T genes

Plasmids encoding CD4 868C or CD4 868T genes had previously been constructed and cloned into a mammalian expression vector. The plasmids were used to transfect A2.01 cell line. Antibiotic resistant A2.01 cells expressing CD4 molecules were selected for by culturing the cells under selection media (G418, 1mg/ml) and tested for CD4 expression by flow cytometry. A2.01/868C and A2.01/868T cells expressing similar levels of CD4 molecules were selected for by cell sorting under sterile conditions using fluorescence activated cell sorting (Fig 9). Epics Elite (Coulter) flow cytometer was used to sort bulk populations expressing comparable levels of CD4 molecules. Limiting dilution method was used to select for A2.01/868C and A2.01/868T clones expression similar levels of CD4 isoforms. Both A2.01/868T and A2.01/868C cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin, streptomycin and G418 (1mg/ml).
3.6.3 Confirmation of the CD4 868C and 868T genes in the transfected A2.01 cells

DNA Isolation

DNA for PCR and sequencing procedures was isolated from $2 \times 10^6$ of A2.01, A2.01 868C, and A2.01 868T cells using a QIAamp DNA Mini Kit (Qiagen). In brief, the cells were washed in PBS, pelleted and resuspended in 200 μl of PBS. In order to lyse the cells, 20 μl of Proteinase K (920mg/ml) was added to the pellet and mixed gently. To achieve complete cell lysis, additional 200μl of buffer AL (lysis solution) was added to the cell lysate, followed by a brief spin for 6 seconds and incubation in waterbath at 37°C for 56 minutes. The cell lysate was transferred into QIAamp mini spin columns where they were given a quick spin to enable DNA to bind onto silica membrane. This was followed with two washes, first with buffer AW1 and a second wash with buffer AW2 to remove any residual contaminants from the cell lysate. The membrane bound DNA was eluted by running through elusion buffer, Buffer AE at room temperature and the purified DNA collected in elusion buffer AE and stored at -20°C.

CD4 sequence analysis confirmatory Assay

The isolated DNA samples were first PCR amplified in the region of the CD4 gene containing the nucleotide position 868. The PCR procedure was performed using a master mix of 5 μl of Invitrogen 10x Mg- PCR buffer, 1.5 μl of Invitrogen™ 50mM MgCl₂, 8.0 ul of dNTP, 28.25 μl of double distilled autoclaved water, 0.25μl of Invitrogen™ recombinant Taq DNA polymerase and 5.0 μl of a 5μM primer mix of primer 5N and 3N (Table 1) per each reaction. Each reaction tube contained 48 μl of this mix and 2μl of the desired DNA template. An approximately 300 base pair region was amplified using the primers listed in Table 1. The first round PCR was performed on an MJ Research PTC-200 thermal cycler under the following conditions: 94°C for
2 minutes, followed by 30 cycles of 94°C for 30 seconds, 64°C for 30 seconds and 72°C for 30 seconds with a final elongation of 72°C for 7 minutes. Each individual sample was amplified in the presence of primers in a reaction tube.

The PCR products were then purified using Millipore Microcon® PCR Centrifugal Filter Devices. Each filter device was inserted into a collection tube provided in each kit. Four hundred and fifty microliters of pH 8.0 Tris-EDTA (TE) buffer was added to each filter device followed by 50 µl of PCR product. The apparatus was then centrifuged at 1000xg for 15 minutes in a table top microfuge (Fisher IEC Micromax). In order to recover the DNA, 20µl of deionized distilled, autoclaved water was added to each filter device and the device was placed upside down into a clean collection 1.5ml eppendorf tube. The eppendorf tubes were centrifuged for 2 minutes at 1000g. These purified samples were then stored at −20°C.
<table>
<thead>
<tr>
<th>Primer name and location</th>
<th>Primer sequence</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' outer (nt 737-757)</td>
<td>5' CAAAATAGACATCGTGTTGCT 3'</td>
<td>PCR amplification</td>
</tr>
<tr>
<td>5N(nt 812-833)</td>
<td>5' GTTCTCCTCCCACTCGCCTT 3'</td>
<td>forward sequence/PCR</td>
</tr>
<tr>
<td>3N(nt 1049-1071)</td>
<td>5' TTCCTGTTTTCGTTCAAGGGCC 3'</td>
<td>reverse sequence</td>
</tr>
<tr>
<td>3' outer (nt 1072-1095)</td>
<td>5' CCAGGTTTCACTTCTGATGCAAC 3'</td>
<td>PCR amplification</td>
</tr>
</tbody>
</table>
Cycle sequencing was then performed on these PCR products using Applied Biosystems PRISM® Big Dye™ Terminator Mix version 3.0 which contains fluorescently labeled dideoxynucleotide triphosphates, appropriate salt concentrations and high fidelity sequencing grade AmpliTaq® DNA polymerase. Each reaction tube contained 2 µl of Terminator mix, 1.5 µl of 3.2pmol/µl primer, 1.0 µl of double distilled autoclaved water and 1 µl of DNA template (the purified PCR amplified region of CD4). The primers used to generate both forward (5N) and reverse (3N) sequences are listed in Table 1. The cycle sequencing procedure was conducted on an MJ Research PTC-200 thermal cycler under the following conditions: 96°C for 3 minutes followed by 60 cycles of 96°C for 30 seconds, 50°C for 30 seconds and 60°C for 4 minutes.

The amplified samples were precipitated using a NaOAc - ethanol method. To each cycle sequenced reaction, 1 µl of 3M NaOAc pH 5.2 was added followed by 3x volume of 95% ethanol. The samples were left to precipitate overnight at room temperature. The samples were then centrifuged a maximum speed for 30 minutes. The pellet was then washed with 200µl of 70% ethanol and centrifuged for 15 minutes. The pellet was then dried and resuspended in 20 µl of high grade formamide. The samples were sequenced on an Applied Biosystems 3100 Automated Sequencer and analyzed using Applied Biosystems Sequence Analysis software. The samples were sequenced using a 20 second injection time and the BC-3100_seqoffFlOFF analysis module. Heterozygotes were identified by the appearance of both a C and a T peak overlapping at position 868 (Figure 10).
3.6.6 Purification of anti-CD4 (SIM4)

Protein G Column (Sigma Alrich, Saint Louis, USA) was used for SIM.4 antibody purification. The columns were equilibrated with binding buffer (15-30ml). The culture supernatant from SIM.4 hybridoma was charged through the column. The column was washed with binding buffer and the antibody eluted into a 1ml solution into tubes by running through Elution buffer. The tubes used for collecting the eluted solution contained Tris-HCl pH 9.0 to neutralize the pH. The eluted solutions from all the tubes were pooled into a single tube and dialyzed overnight in a dialyzing solution. The antibody concentration was determined by BIORAD Bradford protein quantification assay.

3.6.7 Staining A2.01/868T and A2.01/868C cells for CD4 expression using anti-OKT4

Five microliters of anti-OKT4 monoclonal antibodies was used to stain 1 x 10^6 of A2.01, 868C and 868T cells. The cells washed to remove any unbound antibody, followed by staining with an anti-mouse PE (secondary antibody) and analyzed by flow cytometry. Anti-OKT4 binds to an epitope on the third domain (D3) of CD4 where the CD4 SNP occurs. The CD4 expression as determined by OKT4 is shown on figure 11.

3.6.8 Isolation of PBMCs from Whole blood

Twenty to thirty milliliters of whole blood collected in heparinized vacutainer tubes were centrifuged using a bench top centrifuge (Allegra 6R Centrifuge, Beckman Coulter) at 400g for 10 minutes at room temperature. Plasma was removed and the remaining blood diluted with equal volumes of sterile PBS. Ten milliliters of Ficol (Lymphoprep, MJS Biolynx Inc.) was added to a 50ml conical Falcon tube and the diluted blood carefully layered onto to it. Tubes
were centrifuged for 25 minutes at room temperature with brakes off. After centrifugation the diluted plasma on top of the cloudy interface (PBMC layer) was removed and discarded. The PBMC layer was removed and transferred into appropriately labeled 50 ml conical tubes. The cells were washed by filling in tubes with sterile PBS and centrifuged at 1400rpm for 10 minutes with brakes on low. The pellet was resuspended in 20 ml of sterile PBS and 50μl aliquote taken and added into a 96-well round bottom plate for cell counting. For cell counting, 50μl of trypan blue (Sigma) stain was added to the micro-well with the cells, mixed and the mixture charged into a hemocytometer. Cells were counted in all the four squares and their concentration determined. PBMCs were washed for the second time and were used immediately for ex vivo assays.
3.7 Specific methods

3.7.1 Binding affinity studies

(i) General experiment set-up

A solution of 2μg/ml of sCD4 (Trinity Biotech, USA) was prepared in coating buffer and 50μl added to all the wells of a flat bottom 96-well plates. The coated plates were incubated at 4°C overnight. The plates were washed once with blocking buffer and either used immediately or frozen at −80°C.

A2.01, A2.01/868C and A2.01/C868T cells were washed with PBS and 1 x 10⁶ cells added to each well and mixed with serial dilutions of biotin conjugated gp120 starting from 5nM to 1.25nM. The mixtures were incubated at 4°C for 2 hours to attain equilibrium. Following incubation cells were centrifuged at 1200rpm for 5 minutes in bench top centrifuge. The supernatant was removed and transferred into sCD4 pre-coated 96-well plate for ELISA and the cells were left for staining for flow cytometry analysis.

(ii) Binding affinity studies-ELISA method.

Before adding the supernatant from the binding affinity experiment, the sCD4 coated plates were incubated with blocking buffer for 1 hour at room temperature. Pre-diluted biotin conjugated gp120 was added into the plates for the determination of standard curve. The plates were incubated at 4°C overnight. The plates were washed 5 times with wash buffer to remove any unbound gp120, and 100μl of diluted SAAP added to all the wells. The plates were incubated at 37°C for 1 hour and washed. One tablet of phosphatase substrate (Sigma, catalogue number 104105) was diluted with 5ml of DAE solution. One hundred microliters of the diluted substrate
was added to all wells and incubated at 37°C for 1 hour. The plates were read at 405 nm using SPECTRA-max plus (Molecular Devices).

(iii) Binding affinity studies-Flow cytometry method.

The left-over cells from the previous section were stained with 3 μl of SAA-PE (BD Biosciences) and incubated for 30 minutes at 4°C. They were washed with PBS with 2% FCS and analyzed using flow cytometer (BD Biosciences).
3.7.2 HIV infection studies

(a) HIV infection studies using CD4 dependent virus.

(i) Generating HIV stocks.

PHA stimulation of PBMCs

Whole blood was obtained from HIV uninfected individuals from Winnipeg, Manitoba. These individuals were CD4 genotyped and were divided into two groups based on their CD4 868 allele. While the use of people with homozygous for the CD4 polymorphism would be preferred, these individuals are very rare and as such were not available for these studies. PBMCs were obtained using ficol paque method as described in the methods section above. The PBMCs were set up at $2 \times 10^6$ cells/ml, in a volume of 20 ml of RPMI 1640 with 5μg/ml of PHA (Sigma) per flask. The cultures were incubated for 3 days at 37°C, 5% CO$_2$ with humidity.

Propagation of HIV viral stocks in PHA stimulated PBMCs

Laboratory adapted viruses HIV-1$_{M1B}$, HIV-1$_{BAL}$ and primary Kenyan viral isolates, ml235 and ml1956 were propagated in PHA stimulated PBMCs of HIV-1 seronegative normal blood donors. In brief, a vial of each stock of virus was thawed quickly in water bath set at 37°C and quickly transferred into a 75ml tissue culture flask containing 20ml of $5 \times 10^6$/ml of PBMC in growth media. PBMCs/virus cultures were cultured at 37°C in a 5% CO$_2$ with humidity for 10 days for the laboratory adapted HIV viruses and 14 days for the Kenyan viral isolates respectively. The cultures were harvested, an aliquot used for HIV-1 p24 ELISA and the remaining culture supernatant frozen at -80°C in 1ml aliquots. Using a set of fresh PHA stimulated PBMCs, a 50% Tissue Culture Infectious Dose (TCID$_{50}$) experiment was set up. In
briefly, 50μl of 5 x 10⁶ cells/ml of PBMCs were added into a 96-well tissue culture plate. The frozen viral stocks were thawed quickly and titrated against the cell suspension. The cultures were incubated for 6 days for the laboratory adapted viruses and for 10 days for the Kenyan viral isolates. The supernatant were harvested, inactivated by adding 1% triton X (Sigma) detergent to all wells. Using a gag p24 ELISA the amounts of HIV-1 p24 antigen produced were determined and TCID₅₀ calculated. The next section illustrates an example of TCID₅₀ calculation.

(ii) Calculation of virus titres.

*Calculation of TCID₅₀*

In order to determine the TCID₅₀ of the virus stock of each of the viruses used for infection assays, the HIV-1 p24 data obtained from infection of PBMCs were used. The TCID₅₀ was calculated using the method of Reed and Muench (Dulbecco 1988). As a theoretical example, table 3 and table 4 will serve as typical examples of how a p24 ELISA results were used to calculate TCID₅₀. In brief, vertical rows of wells that contain both infected and uninfected cells were identified (Table 3). In order to identify the range of dilutions containing the 50% endpoint dilution, the nearest dilution that contain either all infected wells (4⁻⁶ dilution) and all uninfected (4⁻⁸ dilution) was determined as the dilution from 4⁻⁶ to 4⁻⁸. This dilution was used to calculate the TCID₅₀ from the cumulative values (Table 4). Based on the data in table 4, the 50% endpoint dilution lies between 4⁻⁷ and 4⁻⁸. To determine the 50% endpoint(y) the following formula was used.

\[
y = \frac{(% \text{Mortality at dilution next above 50%}) \text{ minus 50%}}{(% \text{mortality at dilution next above 50%}) \text{ minus } (% \text{ Mortality at dilution next above 50%})}
\]
\[ y = \frac{66.7 - 50\%}{66.7 - 0\%} \]

\[ y = 0.25 \]

The 50% endpoint dilution is \(4^{(7+0.25)}\) or \(4^{7.25}\)

To convert \(4^{7.25}\) into \(10^x\) the following equation was used.

\[ x = (-7.25) \times \log 4 \]

\[ x = (-7.25) \times 0.602 \]

\[ x = -4.365 \]

Therefore, the 50% endpoint dilution in this example was estimated at \(10^{-4.365}\) and the TCID\(_{50}\) at \(10^{4.365}\) Infectious Units (IU).

The TCID\(_{50}\) obtained above was converted into TCID\(_{50}/\text{ml}\) as follows;

\[ \text{xTCID}_{50} \text{IU/ml} = 10^{4.365} \]

\[ 0.05 \text{ml (the volume of virus stock/well)} \]

\[ x = 20 \times 10^{4.365} \]

\[ x = 10^{1.301} \times 10^{4.365} \]

\[ x = 10^{5.666} \]

Therefore, in this example \(10^{5.666}\) TCID\(_{50}\)IU/ml or \(4.63 \times 10^5\) TCID\(_{50}\)IU/ml was the TCID\(_{50}\) based on the data obtained from table 4. The TCID\(_{50}\) obtained was used to calculate multiplicity of infection as follows.

**Calculation of multiplicity of infection (MOI)**

The MOI used in this study was calculated by dividing TCID\(_{50}\) of the virus by the number of cells used for infection. For example, 1 ml of HIV-1 stock containing \(2 \times 10^6\)IU/ml used to infect \(1 \times 10^6\) PBMCS gives MOI of 2 IU/ml.
Table 3: A layout of HIV-1 infection results for calculating TCID50. The table below shows an example of a typical 6 or 10 days p24 results obtained from supernatant of PBMCs infected with different virus dilutions($4^{-1}$ to $4^{-11}$). The negative control consisted of cells and media. The wells positive for HIV-1 p24 are represented by + (infected) and negative by – (uninfected).

<table>
<thead>
<tr>
<th>media</th>
<th>$4^{-1}$</th>
<th>$4^{-2}$</th>
<th>$4^{-3}$</th>
<th>$4^{-4}$</th>
<th>$4^{-5}$</th>
<th>$4^{-6}$</th>
<th>$4^{-7}$</th>
<th>$4^{-8}$</th>
<th>$4^{-9}$</th>
<th>$4^{-10}$</th>
<th>$4^{-11}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
Table 4: Tabulation of HIV-1 infection results for calculation of TCID50. The example below is a table with three different HIV-1 dilutions and accumulative HIV results used to select the dilutions for the calculation TCID50.

<table>
<thead>
<tr>
<th>Dilutions</th>
<th>Infected</th>
<th>Uninfected</th>
<th>Total infected</th>
<th>Total uninfected</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>$4^{-6}$</td>
<td>6</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>10/10</td>
</tr>
<tr>
<td>$4^{-7}$</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>4/6</td>
</tr>
<tr>
<td>$4^{-8}$</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>8</td>
<td>0/8</td>
</tr>
</tbody>
</table>
(iii) HIV infection-Experiment set-up.

In this study HIV infections were done at both in vitro and ex vivo levels. For in vitro studies, HIV-1$_{IIIb}$, ml1956 viruses were used to infect A2.01, A2.01 868C and A2.01 868T cells. However, for ex vivo studies, HIV-1$_{BAL}$ virus was used to infect unstimulated PBMCs. For both PBMCs and cell lines infection assay, 2 x 10$^6$ cells were used. The cells were spun down, supernatant removed, appropriate amount of viral stock suspension added and incubated for 3 hours at 37°C with constant mixing after every 30 minutes. The amount of virus used for these assays were an MOI of 0.01 and 0.001 for Kenyan viral isolates and laboratory adapted viruses respectively. After virus adsorption, the cells were washed twice with cold RPMI 1640 with 10% FCS, resuspended in growth media and incubated for six days at 37°C. A2.01 cells were used as a mock infection whereas for ex vivo infection PBMCs and media served as a negative control. Culture supernatants and cells were harvested on days 1, 3 and 6 post infections and HIV-1 infection was monitored by quantification of HIV-1 p24 antigen by HIV-1 p24 ELISA and intracellular staining for HIV-1 p24 by flow cytometry respectively.

(iv) HIV p24 ELISA method

Monoclonal anti-p24 was diluted in coupling buffer to 2μg/ml and 100ul added to all the wells of NUNC-maxisorb 96 well ELISA plate. The plates were incubated at either 37°C for 3 hours or overnight at 4°C. The supernatant were removed and the plates either stored at -70°C until needed or used immediately for detection of HIV p24 antigen from the culture supernatant. Frozen plates were thawed and 100μl of blocking buffer added to each well and incubated for 1 hour at room temperature. The plates were washed with PBS-T20 to remove any unbound excess blocking buffer. The culture supernatants that had been treated with 10% triton-x-100(final is
1% were added to each well. At the same time standard control samples were added in doubling dilutions from 20µg/ml (10µl Supt-Eth stock). This was followed with overnight incubation at 4°C. Plates were washed to remove any unbound HIV-1 p24 antigen from the culture supernatant and 100ul/well rabbit polyclonal anti-p24 (final dilution of 1:16,666 in blocking buffer) added. The plates were incubated with rabbit polyclonal anti-p24 was at 37°C for 90 minutes to allow the anti-p24 antibody to bind to p24 antigen. This was followed with a wash to remove excess anti-p24 polyclonal antibody. Biotin anti-rabbit (final dilution of 1:28,750 in blocking buffer) was added to the plate followed with 60 minutes incubation at 37°C. At the end of the incubation the plate was washed and 100/well SAAP (final dilution of 1:13,333 in blocking buffer) added to all wells and plate incubated at room temperature for 60 minutes. The last wash was done five times with PBS-T20 followed by addition of 100ul/well substrate and incubation at 37°C for 60 minutes. The plates were read at 405 nm (550 reference).

(v) Detection of intracellular HIV p24 antigen by flow cytometer.

Cells that had been infected with HIV for 3 and 6 days in a 96-well plate were fixed by adding 175µl of BD Fix/Perm solution and incubated for 20 minutes at 4°C. A volume of 1.5ml Perm Wash (1x) buffer was added to each tube and spanned down at 1600rpm for 10 minutes. The supernatant was poured off and the cell pellet resuspended. This was followed with addition of 2µl (10µg/ml) of KC57-FITC (Anti-p24) and incubation for 30 minutes at 4°C. The cells were washed twice with cold PBS and resuspended in 300ul of PBS. The cells were analyzed by use of FASCalibur (BD Biosciences).
(b) HIV infection studies using CD4 independent viruses

(i) Luciferase assay-Method

A total of $1.8 \times 10^6$ cells per tube of A201, 868C and 868T cells were exposed to 24.8μl (2cpm/cell) of HIV-1 PNL 4.3-Luc+/Env/VSV-G+ virus for 2 hours at 37°C with mixing after every 30 minutes. The cells were washed twice with RPMI 1640 media, resuspended in fresh media, aliquoted at $2 \times 10^5$ cells per well and incubated at 37°C for 48 hours. Supernatants were collected for HIV-1 p24 antigen detection and cells for Luciferace assay.

(ii) Sample preparation and analysis

HIV-1 infected cells were collected and washed after 48hrs of incubation. Cell lysis buffer was prepared by adding 4 volumes of dH2O to 1 volume of 5x lysis buffer and 50μl dispensed into micro centrifuge tube containing $1 \times 10^5$ cells. The cell pellet from each tube was vortexted for 10-15 seconds and centrifuged at 12,000 x g for 2 minutes at 4°C. The supernatant was transferred to a new tube. Added 20μl of room temperature cell lysate to plate wells. Fifty microlitres of Luciferase Assay substrate was added to each well and the amount of light measured immediately using Luminometer (Model, LB 96V).

3.7.3 Signal Transduction studies.

(i) Western Blot.

A2.01, A2.01 868C and A2.01 868T cells were washed in serum-free Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen). The CD4-negative A2.01 cells were used as a negative control. One milliliter of cell suspensions @ $10^6$/ml, were added to 4ml Falcon tubes, the tubes spun down, supernatant poured off and 10μg/ml of anti-CD4 (SIM4) added to each tube. Cells
and antibody were incubated at 4°C to allow anti-CD4 bind to CD4 molecules on the cells, this was followed with a wash to remove excess anti-CD4 and addition of 5μl (10μg/ml) of goat anti-mouse (GAM) for cross-linking anti-CD4. The cells were lysed by adding cell lysis buffer and incubating for 20 minutes at 4°C. The cell lysate was transferred to 2 ml microfuge tubes and spun for 15 minutes at 4°C to clear the lysate of cell debris.

Coating Protein A agarose beads with anti-CD4 and anti-lck

Fifty microliters of protein A agarose beads (Sigma Aldrich, USA) slurry was added into an eppendorf tube. The beads were washed twice by addition of 450μl of ice-cold lysis buffer and spun for 10 seconds at 1000rpm in a microfuge (Microspin 24S, Sorvall Instruments). Four microgram per milliliter of either anti-CD4 mAb or anti-lck mAb was added to a separate microfuge tube containing 60μl of washed protein A agarose bead slurry. The tubes were rocked at 4°C for 1 hour and collected by centrifugation for 5 minutes in a microfuge. The beads were washed three times with ice-cold PBS and the supernatant aspirated leaving 50μl of the antibody coupled beads suspension.

Immunoprecipitation (IP)

Five hundred microlitre of cell lysate was added to the antibody coupled beads and mixture incubated at 4°C for 2 hours. The complex consisting of beads/antibody/ protein was washed three times with ice-cold lysis buffer. The beads/protein complex was resuspended in sample buffer and boiled for 5 minutes in a water bath.
ImmunoBlot Protocol

A separating SDS-Page Gel (10%) was prepared as described under solution and reagents for western blot. The separating gel was charged into the Gel apparatus and left to solidify at room temperature for 1 hour. Ten minutes to the end of incubation, Stacking Gel was prepared as described under “solutions and reagents” for western blot above. The stacking gel was attached to the apparatus and gel combs loaded into the gel and incubated for 1 hour at room temperature. The combs were carefully removed and running buffer added to the wells to fill-in for any air bubbles. Electrophoresis tank was set-up by transferring the gel apparatus into the tank and adding 1x Running Buffer to the required level. Twenty micro-liters of boiled beads/protein complex and 5ul of molecular marker were added to their respective wells. The electrophoresis apparatus was run at 190 volts for 55 minutes.

The gel was removed from the electrophoretic tank and equilibrated in transfer buffer for 60 minutes. Nitrocellulose paper (Sigma, Catalogue number N7892) was cut and soaked in methanol for 10 minutes and another 10 minutes in transfer buffer respectively. Immunoblot sandwich was set-up in the following order; pre-wet filter paper/ pre-wet nitrocellulose membrane/ gel/ pre-wet filter paper. The electrophoretic tank was set to run at 15volts for 45 minutes. The blotted nitrocellulose was transferred into a container with blocking solution and incubated for 60 minutes at room temperature and washed once with wash buffer. Primary antibodies (anti-CD4 mAb and anti-Ick mAb) were added at 1ug/ml in blocking solution and incubated at 4°C overnight with constant agitation. The blots were washed six times with wash buffer at 5 minutes incubation per wash and then blocked for 1 hour in blocking buffer. Secondary antibodies were prepared at 1:20,000 dilutions with blocking buffer. For the detection
of p56\textsuperscript{ck}, anti-rabbit conjugated with Horseradish Peroxidase (HRP) was used. Goat anti-mouse conjugated HRP was used for the detection of CD4 and phosphorylated p56\textsuperscript{ck} respectively. The blotting paper was incubated in the presence of secondary antibodies for 1 hour at room temperature with constant agitation. This was followed by 6 washes with wash buffer. Enhanced chemiluminescent (ECL) kit (Amersham Biosciences) was used for the visualization of bands. In brief, equal volumes of ECL Advance Solution A and Solution B were added into a 5 ml Falcon tube, mixed and kept away from light. The solution was poured onto a nitrocellulose covered with a polythene paper and the bands visualized by a densitometer.

(ii) Luminex assay

To minimize cell stimulation from Fetal Calf Serum (FCS), \(10 \times 10^6\) of A2.01/868C and A2.01/868T cells expressing similar levels of CD4 were starved overnight by culturing in RPMI with 2% FCS. The cells were incubated with either with 10\(\mu\)g of anti-CD4 (SI-M4) or with media alone for 45 minutes at 4\(^\circ\)C. The cells were washed twice and cross-linked by adding 10\(\mu\)g of goat anti mouse and incubating for 10 minutes at 4\(^\circ\)C and 3 minutes at 37\(^\circ\)C. The cells were lysed using Upstate beadlyte cell signaling lysis buffer and cell lysate clarified by centrifugation. Cell lysate was diluted 1:10 in PBS and protein concentration determined by Bradford method. Detection of phosphorylated p56\textsuperscript{ck} was done using Upstate Beadlyte cell signaling assay kit. In brief, 25\(\mu\)g of cell lysate from stimulated and unstimulated cells were added to a pre-wetted 96 well filter plate containing anti-lck coated beads and incubated overnight at 4\(^\circ\)C. The plates were washed and a conjugate and substrate added to each well. The mean fluorescence emitted was read using Luminex 100\textsuperscript{TM} system.
(iii) *Flow cytometry*

A total of $2 \times 10^6$ of 868T and 868C cells expressing similar levels of CD4 were stimulated as described above. The cells were fixed and permeabilized using cytofix/cytoperm reagents (BD Bioscience) and stained with 10μl of Phycoerythrin (PE) conjugated anti-Lck pY505 (BD Biosciences, Catalogue number 558552) for 20 minutes at 4°C. Cells were washed and analyzed using FASCalibur (BD Bioscience).

### 3.8 Statistics calculation

All the data generated in this study were analyzed using student $t$ test.
5.0. Results

5.1 Developing cell lines for *in vitro* assay

*Rationale*

Epidemiologic association studies on CD4 polymorphism and HIV/AIDS have demonstrated that having this genetic variant is a risk factor for both HIV acquisition and disease progression. While such studies are informative, the associations observed between a genetic variant and a disease condition may or may not hold true. As such further studies using *in vitro* system are always required to confirm the association. It is for this reason that we developed cell lines that resemble CD4 +T cells with either CD4 868T and CD4 868C alleles.

*Results*

As outlined in the materials and methods section of this thesis, the A2.01 cell line was chosen for *in vitro* assay. The cells had been previously transfected with genes to CD4 868T and CD4 868C by Dr. Judie Alimonte, a postdoctoral fellow in Dr. Fowke’s laboratory. Subsequently, A2.01, A2.01/868T and A2.01/868C cells were stained with anti-CD4 PE (Lue-3a) to determine CD4 expression levels on both A2.01/868T and A2.01/868C cells. As shown in (Figure 9a), these cells had two peaks each and the CD4 expression on both cell types were different. In order to obtain cells expressing similar levels of CD4, the cells were processed by cell sorting. In brief, 80 million cells from each cell line were stained with anti-CD4 PE and then sorted using a Coulter flow cytometer. The sorted cells were cultured for two weeks and stained for CD4 expression. As shown in figure 9b, A2.01/868T and A2.01/868C cells had similar CD4 expression.
By selecting for A2.01/868T and A2.01/868C cells that express similar levels of CD4, we were able to remove one of the confounding factors, the differences in CD4 expression between the two cell lines.
Figure 9: CD4 Expression on 868T, 868C before and after cell sorting. A2.01, 868T and 868C cells were stained with anti-CD4 PE immediately after transfection (A) and after cell sorting (B). A2.01, 868T and 868C cells are represented by blue, green and pink curves, respectively. Cell sorting was used to select for cells expressing similar levels of both wild type CD4 and CD4-Trp240(868T).
While selecting for cells that express similar levels of CD4 was an important step, there was a need to confirm that these cells were different with respect to their genotype. In order to confirm the differences in CD4 genotype, the CD4 gene was sequenced and their CD4 genotype determined. As shown in figure 10, both A2.01 and cells expressing wild type CD4 were confirmed to have wild-type CD4 genotype. On the other hand, cells expressing mutant CD4 were confirmed to contain the mutant gene (figure 10).
Figure 10: Confirmation of 868T and 868C genes in the transfected A2.01 cells. Cells transfected with either 868C or 868T CD4 genes were lysed and PCR sequenced using primers targeting the CD4 SNP. A2.01 cells were included as a control. This figure shows that A2.01 cells lack the expression of CD4 but have genomic genes for the wild type CD4 gene, A2.01/868C cells have the wild-type CD4 gene and A2.01/868T cells have both wild type and polymorphic CD4 genes.
So far we have successfully developed A2.01 cells that express similar levels of both wild type and mutant CD4 and have confirmed their genotypes. However, before using the cells for functional assays, we wanted to investigate if OKT4 epitope on the mutant CD4 molecules has been changed as has been reported previously. These studies had shown that monoclonal anti-OKT4 does not bind to the mutant CD4 molecule but binds to wild-type CD4 molecule (Fuller TC, 1984). OKT4 epitope is found on the third domain (D3) of CD4 molecule where the CD4 mutation (868T) also occurs. In order to confirm that CD4 molecules on A2.01/868T cells resemble those that have been described in individuals with CD4 868T, A2.01, A2.01/868T and A2.01/868C cells were stained with anti-OKT4. As shown in figure 11, A2.01 and A2.01/868T cells stained negative with anti-OKT4, while A2.01/868C cells stained positive with anti-OKT4. These results suggest that the CD4 molecule on the mutant CD4 has an altered epitope at least at the OKT4 binding site.

Taken together, these results have confirmed to us that the cell lines we have developed resemble those found in individuals with either CD4 868T and CD4 868C alleles and as such were suitable for use in our in vitro functional assays.
Figure 11: Staining of A2.01/868T and A2.01/868C cells with anti-OKT4. A2.01, A2.01/868C and A2.01/868T cells were stained with anti-OKT4. A2.01 cells were used as a negative control. A2.01 cells, A2.01/868T and A2.01/868C cells are represented by blue, pink and green curves respectively. These results show that anti-OKT4 an antibody that binds on the third domain of CD4 only binds to CD4 molecules on A2.01/868C cells and not A2.01/868T cells as been shown in previous publication (Fuller TC, 1984).
5.2 CD4 polymorphism and susceptibility to HIV-1 infection.

5.2.1 Evaluating susceptibility of A2.01/868T cells to infection with HIV viruses.

Rationale

Epidemiological studies done by our group among the commercial sex workers at the Pumwani Majengo sex workers cohort in Nairobi, Kenya found that HIV negative women who have CD4 C868T when enrolled in our studies were more likely to seroconvert to HIV-1 sooner than their counterparts with CD4 868C (Figure 7). It is not known whether this enhanced susceptibility to HIV infection in these women could be because their CD4+T cells are more easily infected by HIV or there are other unknown factors involved. Susceptibility to HIV-1 infection can be determined by host factors as well as other factors such as the presence of a sexually transmitted infection. Since these women were commercial sex workers and had a similar high level of exposure to HIV-1 (Françoise Vouriot, Thesis, 2005), we sought to investigate if their difference in susceptibility could be due the differences in CD4 phenotypes.

Hypothesis

A2.01/868T cells are more susceptible to HIV infection than A2.01/868C cells.

Objective

To investigate if the differences in susceptibility to HIV-1 infection in cell lines with either CD4 868T or CD4 868C allele is at the cellular level.
**Study outline**

A2.01, A2.01/868T and A2.01/868C cells were infected with HIV-1$_{11B}$ virus at an MOI of 0.001 or with a primary HIV-1 isolates at an MOI of 0.01. Following 3 hours incubation with virus, the cells were washed, re-suspended in media and incubated for 6 days. Culture supernatants were obtained on days 1, 3 and 6. HIV-1 p24 ELISA was used to determine the amount of p24 antigen in the culture supernatant. On day 6, the cells were harvested and stained for the presence of intracellular p24 antigen using anti-p24 FITC (KC67). The cells were washed and analyzed by a flow cytometer.

**Results**

Comparison of the amount of HIV-1 p24 antigen detected in the culture supernatant of HIV-1$_{11B}$ infected cell cultures, revealed that on day 3 the amount of HIV-1 p24 antigen was significantly higher from A2.01/868T infected cells than A2.01/868C infected cells (Figure 12, **p=0.026).** Further incubation of the cultures to day 6, showed a consistent increase of HIV-1 p24 antigen from culture supernatant of A2.01/868T cells than from A2.01/868C cells (Figure 12, ***p=0.0001). In order to confirm whether the cells were infected, on day 6 post infection, the cells were harvested and stained for intracellular HIV-1 p24 antigen. Results from this experiments show that about 7.3% of A2.01/868T cells had detectable intracellular p24 compared to 0.37% of A2.01/868C cells (Figure 13). The percentage of HIV infected cells from this assay was quite low, suggesting that flow cytometry has a lower sensitivity compared to ELISA method. However, both ELISA and flow cytometry results suggest that A2.01/868T cells are more susceptible to HIV infection than A2.01/868C cells.
Figure 12: CD4-Trp240(868T) expressing cells are more susceptible to infection with HIV-1<sub>IIIB</sub> virus. A201 cells expressing equivalent levels of either wild type CD4 (CD4wt) or CD4-Trp240(868T) were infected with HIV-1<sub>IIIB</sub> virus at MOI of 0.001. Mock infection consisted of A2.01 cells and virus. The cultures were incubated for 3 hours at 37°C. The cells were washed twice, re-suspended in media and incubated for six days at 37°C. Supernatants and cells were harvested on days 1, 3 and 6 and tested for HIV-1 p24 antigen. For Day1 infections, the differences in the amount of p24 between wild-type and CD4-Trp240(868T) were statistically not significant (NS). The bars represent the mean p24 antigen production of 6 replicate wells with the error bars representing standard deviation of the mean. This experiment was repeated three times with similar results.
Figure 13: Determination of HIV-1 infected cells by flow cytometry. A2.01/868T and A2.01/868C cells expressing equivalent levels of CD4 were infected with HIV-1 
IIIb virus at MOI of 0.001. The cultures were incubated for 3 hours at 37°C. The cells were washed twice, re-
suspended in media and incubated for 6 days at 37°C. The cells were stained with a FITC labeled
an anti-p24 (KC67), analyzed by flow cytometry and the percentage of p24 positive cells
determined by cells quest pro software. A higher percentage (7.3%) of A2.01/868T cells were
positive for HIV-1 p24 compared to about 0.37% of A2.01/868C cells. This experiment was
repeated twice with similar results.
While the use of laboratory adapted virus such as HIV-1\textsubscript{HIV} is common for \textit{in vitro} studies, these viruses have enhanced infectivity compared to the viruses that circulate in human populations. This characteristic of laboratory adapted viruses prompted us to explore primary viral isolate. Subsequently, a primary viral isolate, ml 1956 virus was used for infection. Results obtained from day 6 post infection, show that a significantly higher amounts of HIV-1 p24 antigen was detected from culture supernatants of A2.01/868T cells than A2.01/868C cells (Fig14, p=0.0011). Overall these results suggest that CD4-Trp240(868T) expressing cells are more susceptible to infection with both laboratory adapted virus HIV-1\textsubscript{HIV} virus and primary viral isolates compared to cells that express wild type CD4.
Figure 14: Infection of A2.01, A2.01/868T (Trp240) and A2.01/868C (wt) cells with Kenyan primary HIV-1 viral isolate. A total of 2 x 10^6 of A2.01, A2.01/868T and A2.01/868C cells were infected with Kenyan primary viral isolate ml1956 at an MOI of 0.01 for 3 hours at 37°C. A2.01, the parental cell line was used as a negative control. The cultures were re-suspended in media and cultured for 6 days at 37°C. Supernatants were harvested and virus production was measured by p24-ELISA. The bars represent the means ± SE of HIV-1 p24 antigen production of 6 replicate wells with the error bars representing standard deviation of the mean. This experiment was repeated three times with similar results.
5.2.2 Assessing susceptibility of A2.01/868T and A2.01/868C cells to infection with a CD4-independent HIV-1 virus.

_Rationale_

The HIV-1 infection results from the previous section suggest that the differences in infectivity between the cells expressing either wild type or the variant CD4 isoform could be due to the differences in CD4 phenotypes. However, because the two CD4 isoforms clones used in the previous assay were produced independently, it is possible that the differences in susceptibility to HIV infection could be due to factors associated with the individual clone and not due to the different CD4 phenotypes. In order to clarify this issue, a CD4-independent virus was used for infection of the cells transfected with the two CD4 isoforms.

_Objective_

To investigate if A2.01/868T and A2.01/868C cells expressing similar levels CD4 have different susceptibility to infection with a CD4-independent HIV virus.

_Study outline_

In order to determine if the differences in susceptibility to HIV infection was CD4 phenotype dependent, a CD4-independent HIV pseudotype virus was used for infection. This virus had a backbone of HIV, the _nef_ gene was replaced with luciferase gene and the _env_ gene was knocked out, a plasmid containing the vesicular stomatitis virus glycoprotein (VSV.G) was co-transfected into the stock cells and used to replace gp120. Due to lack of _env_ gene in this virus, it was
expected to cause single round infection. Virus infected cultures were incubated for 48 hours. Both culture supernatants and infected cells were harvested and the amount of HIV-1 p24 antigen determined by p24 ELISA and the number of infected cells measured using Luciferase assay.

Results

Results obtained from culture supernatants of both A2.01/868T and A2.01/868C cells, showed that the amount of HIV-1 p24 detected by ELISA were not statistically significant (Figure15a, p=0.7108). Similarly, the infected cells were lysed and amounts of luciferase measured. Luciferase assay results also showed that similar number of both A2.01/868T and A2.01/868C cells were infected by the CD4-independent HIV-1 (Figure15b, p=0.4112). Taken together, these results suggest that cells with either CD4 isoforms have similar ability to support HIV-1 replication when using a CD4-independent virus. These results, together with the infection results from the previous section suggest that A2.01/868T cells are more susceptible to HIV infection than A2.01/868C cells and the differences in susceptibility between the two cell type is dependant on the CD4 isoform and not another property of the cell lines.
Figure 15: Infection of A2.01/868T and A2.01/868C cells with a CD4-Independent virus. A CD4 independent (pNL4-3-Luc/VSV-G pseudotype) virus was used at 2cpm/cell to infect A2.01/868T and A2.01/868C cells for 3 hours at 37°C. The cells were washed twice, re-suspended in media and incubated at 37°C for 48 hrs. Supernatants were harvested and HIV-1 p24 antigen determined using ELISA (A). At the same time, cells were harvested, lysed and amount of Luciferase quantified (B). Cells and media alone were used as a negative control. The differences in the amount of either HIV-1 p24 antigen or Luciferase detected between A2.01/868T and A2.01/868C cells were not statistically significantly (p=0.7103 and p=0.4112).
5.2.3 Assessing the susceptibility of individuals with CD4 868T/C for HIV-1 infection.

Rationale

While *in vitro* experiments are an excellent model to test for the effect on CD4-Trp240(868T) on cellular susceptibility to HIV infection, *ex vivo* assays using PBMCs isolated from human subjects better represent the natural setting. There are fundamental differences between cell lines and PBMCs. For example, most cell lines either express only CXCR4 or CCR5 but not both, while PBMCs express both CCR5 and CXCR4 (Binley JM, 2004). Since most circulating HIV-1 viruses during primary stages of infection are R5 or CCR5 tropic viruses and the cell lines used in our previous infection studies only expressed CXCR4 and not CCR5, use of PBMCs was warranted.

Hypothesis

Since epidemiological studies on CD4 SNP and disease association have suggested that people with CD4 868T allele are more susceptible to HIV-1 infection, we hypothesize that PBMCs from such persons are more susceptible to infection with HIV-1.

Objective

To investigate if persons with CD4 868T/C and CD4 868C phenotypes also differ in their susceptibility to HIV-1 infection.
**Study outline**

In order to investigate whether CD4 868T is associated with susceptibility to HIV-1 infection at *ex vivo* level, six HIV-1 uninfected blood donors were selected based on their CD4 genotype. Three were homozygous for wild-type CD4 genes and the other three were heterozygous (868T/C) (at the time of this study no donors homozygous for 868T were available). PBMCs were isolated from whole blood obtained by from each donor. To mimic natural infection, the infection assay was done without prior stimulation of the cells with phytohaemagglutinin (PHA). The details of the infection assay can be found under materials and methods.

**Results**

Unlike in the previous experiments with A2.01 based cell lines where HIV-1_{IIIB} was used, in this experiment HIV-1_{BAL} was used instead. HIV-1_{BAL}, being a CCR5 tropic virus, was appropriate for infection PBMCs as most PBMCs express CCR5. Results from infection of PBMCs with HIV-1_{BAL} virus showed that the mean p24 for CD4 868C/T donors was 400pg/ml while the mean p24 for CD4 868CC donors was 140pg/ml. When the two groups were compared, it was found that the amounts of p24 antigen produced by PBMCs from CD4 868C/T donors was significantly higher compared to donors with CD4 868C/C genotype (Figure16, p=0.0033). These results together with *in vitro* experiment further confirm our hypothesis that CD4 868T cells support a more rapid replication of HIV-1. A manuscript reporting these results was recently accepted for publication in the Journal of Infectious Diseases (JID).
Figure 16: HIV-1 infection of PBMCs from donors with either CD4 868T/C (Donor 73, 80 and 83) and CD4 868C (Donor 19, 39 and 74) genotypes. PBMCs were incubated with HIV-1\textsubscript{BAL} virus at MOI of 0.001 for 3 hrs at 37°C, cells were washed re-suspended in media, aliquoted into six wells and incubated for 6 days at 37°C. The negative control consisted of cells cultured with media alone. The supernatant were harvested and amount of HIV-1 p24 antigen measured using an ELISA. All the heterozygous donors had a significantly higher amount of HIV-1 p24 than donors with wild type CD4.
5.3 Assessing if there are differences in binding affinities between gp120 and the CD4s on 868T and 868C cells.

*Rationale*

CD4+T cells from individuals who are homozygous for 868T phenotype have been shown to stain negative with monoclonal anti-OKT4. This an antibody binds to the third domain of CD4. Additionally, the same antibody has been shown to stain each cell with half the intensity in those who are heterozygous (868C/T) and stain with full intensity all CD4 T cells in individuals with wild type CD4 (Fuller TC, 1984). The failure of these antibodies to bind to and stain CD4+T cells in the subjects with 868T suggests that the CD4 SNP has altered the structure of CD4 molecule at this epitope. As outlined in the introduction section of this thesis, this alteration could lead to changes of tertiary structure of CD4 molecule at D1 of CD4 the site where gp120 of HIV binds. This alteration could either enhance or reduce the binding affinity of gp120 to the cells with this altered CD4 structure. Since binding affinity between gp120 and CD4 is an important determinant of susceptibility to HIV-1 infection, we sought to investigate the impact of CD4 SNP on the binding affinity for HIV-1 envelope protein.

*Hypothesis*

The notion that persons with the mutant CD4 are more susceptible to HIV-1 infection than their counterparts with wild-type CD4 suggests that this enhanced susceptibility could be at the cellular level. In particular, the susceptibility could be at the point of virus entry into a host cell. Additionally, the fact that this CD4 SNP has the potential to alter the tertiary structure of CD4 at domain-1 where gp120 binding site resides suggests that this change could enhance the binding
affinity for gp120. It is due to these reasons that we hypothesized that gp120 binds to CD4 Trp240 with enhanced affinity thereby allowing HIV-1 to infect these cells more readily.

Objective
To investigate if gp120 binds to CD4-Trp240(868T) with higher affinity compared to wild-type CD4.

Study outline
Half a million A2.01, A2.01/868T and A2.01/868C cells were incubated with varying concentrations (10nM, 5nM, 2.5nM, and 1.25nM) of HIV-1 gp120 in a 96 v-shaped well plate for 2 hours at 4°C as described in the methods section. The unbound HIV-1 gp120 was harvested and the amount determined using ELISA. Additionally, the cells were harvested and stained for bound gp120. The cells were analyzed by a flow cytometer.

Results
The ELISA results from these assay showed a consistently significant higher amounts of gp120 being detected on the supernatants from CD4 868T cells compared to CD4 868C cell cultures suggesting that more gp120 were bound to wild-type CD4 compared to CD4-Trp240(868T) during the incubation period (p values; 10nM p=0.02, 5nM p=0.005, 2.5nM p=0.004, 1.25nM p=0.01) (Figure10). These results were confirmed by flow cytometry data. According to flow data, about 12% of A2.01/868C cells stained positive for gp120 compared to about 2% of A2.01/868T cells. Further confirming that A2.01/868T cells bind to gp120 with lower affinity compared A2.01/868C cells (Figure 18).
Figure 17: Determination of binding affinity between gp120 and the two CD4 isoforms. The A2.01, A2.01/868C and A2.01/868T cells were washed with PBS and $1 \times 10^6$ cells/well mixed with serial dilutions of gp120 starting from 10nM to 1.25nM. The mixtures were incubated at 4°C for 2 hours to attain equilibrium. Following incubation, cells were centrifuged at 1200rpm in bench top centrifuge. The supernatant was removed and transferred into sCD4 pre-coated 96-well plate for ELISA. The amounts of unbound gp120 were determined. To obtain bound gp120, the unbound gp120 was subtracted from total gp120. A graph was plotted of bound vs. total gp120. A significantly higher amounts of bound gp120 were detected from CD4/gp120 than from CD4-Trp240(868T)/gp120 mixtures (10nM $p=0.02$, 5nM $p=0.005$, 2.5nM $p=0.004$, 1.25nM $p=0.01$).
Figure 18: Determination of binding affinity between gp120 and the two CD4 isoforms by flow cytometry. A2.01/868T and A2.01/868C cells were stained with streptavidin-PE for bound gp120 for 30 minutes at 4°C. Only cells gated in the live population were analyzed for binding. The percentage of CD4 cells staining positive for gp120 were determined by use of flow cytometry. Approximately 12% of A2.01/868C cells stained positive for bound gp120 compared to about 2% of A2.01/868T cells.
5.4 CD4 polymorphism and HIV/AIDS disease progression studies.

5.4.1. Assessing early signaling events in CD4 868T cells that may influence rapid cell death.

Rationale

Signaling through CD4 has been shown to play a role in stabilizing the major histocompatibility class II/T cell receptor complex and at the same time enhancing antigen-driven T cell activation (André Veillette, 1989). In addition, the binding of gp120 to CD4 can induce apoptosis through either CD95/CD95L-dependent cell death pathway or through a Bax-dependent mitochondrial apoptosis pathway (Perfettini, JL, 2005). The Bax-dependent apoptotic pathway requires p56lk activity. On the other hand, HIV-1 is known to infect activated CD4+T cells more readily than resting cells. Indeed, the interaction between CD4 and gp120 of HIV also transduces signals which may lead activation of CD4+T cells. Although epidemiology studies suggests that CD4 868T is associated with rapid HIV-1 disease progression, it is not known whether CD4 signal transduction may play a role in CD4+T cell death. It is for this reason that we sought to investigate a possible role of CD4 specific signal transduction in the death of A2.01/868T and A2.01/868C cells.

Hypothesis

CD4-specific signal transduction plays a role in initiating signals that may be important for apoptosis.
Objectives

- To compare lck activity between stimulated A2.01/868T and A2.01/868C cells.
- To compare the level of lck phosphorylation on tyr505 between stimulated A2.01/868T and A2.01/868C cells.

Study outline

In order to investigate the differences in signal transduction between the two CD4 isoforms, cells expressing similar levels of the CD4 isoforms were stimulated by cross-linking with monoclonal antibodies to CD4. Cross-linking of CD4+T cells with monoclonal antibodies is a well established method that has been used before to mimic the interaction between CD4 from a T cell and MHC class II molecule from antigen presenting cells (Veillette, A, 1989, Collins TL, 1993, Luo Kunxin, 1990). In our case, the antibody (SIM4) chosen for cross-linking binds to the same epitope on CD4 as gp120 and, therefore, would resemble gp120/CD4 interaction. While use of gp120 would have been ideal, commercially available gp120 have different binding affinities to CD4 depending on source of HIV it is derived from. The cells were lysed and the lysate used in western blot and luminex assays to determine the amount of total phosphorylated lck. In a separate experiment, cell were stimulated in a similar way, stained for phosphorylated tyr505 and analyzed by flow cytometry (Figure 21).

Results

Stimulation of the CD4 isoforms resulted in phosphorylation of lck in about 43% the A2.01/868T cells compared to about 34% phosphorylated total lck in the A2.01/868C cells (Figure 19). Similarly, by using Luminex assay, a mean flourecences of about 800 light units
were detected from A2.01/868T cell lysates compared to about 400 from A2.01/868C cell lysate. The differences in mean fluorescence between A2.01/868T and A2.01/868C cell lysates were statistically significantly different (Figure 20, p=0.0001) suggesting that there was more phosphorylated lck in A2.01/868T cells. The mean fluorescence was used as an indirect measure of total phosphorylation of lck in the cell lysates. We chose to use both western blot and Luminex assay for two reasons. First, western blot is standard method for identifying proteins of known molecular weights. Second, luminex was used as additional assay to confirm western blot results.

Although both western blot and luminex assays found more phosphorylated lck from stimulated A2.01/868T cells than A2.01/868C cells, they were unable to distinguish if these differences were due to phosphorylation of tyrosine residues 394(tyr394) and tyrosine residues 505(tyr505). Consequently, A2.01/868T and A2.01/868C cells were stimulated as described above and stained with anti-tyr505. Results from these studies revealed that about 78% of A2.01/868T cells stained positive for phosphorylated tyr505 compared to 23% of from A2.01/868C cells (Figure 21). Taken together, these results suggest that the binding of CD4-Trp240(868T) to gp120 transduce stronger signals that phosphorylate p56\textsuperscript{ck} on tyr505. A higher phosphorylation on Tyr505 may lead to inactivation of p56\textsuperscript{ck} activity which in-turn results in less activation of the cell (figure 6b). See discussion for further clarification of the impact of phosphorylation of the two tyrosines residues on activation of the cell.
Figure 19: Determination of total phosphorylated lck between A2.01/868T and A2.01/868C cells by Western blot. A2.01/868C and A2.01/868T cells were stimulated by cross-linking with anti-CD4 (SIM4). A negative control consisting of unstimulated cells was included. The negative control represented on the graph was an average reading of the two negative controls from A2.01/868T and A2.01/868C cells. Cells were lysed and the amount of total phosphorylated lck measured by Western blot. (A) Lanes 1 and 3 represent the amount of total lck measured from unstimulated A2.01/868T and A2.01/868C cells. Lanes 2 and 4 are the amounts of total phosphorylated lck from stimulated A2.01/868T and A2.01/868C cells respectively. (B) Represents the amounts of total phosphorylated lck as measured by densitophotometer. The amount of total phosphorylated lck in A2.01/868T cells are 42.9% compared 33.6% for A2.01/868C cells. The experiment was repeated three times with similar results.
Figure 20: Determination of phosphorylated lck between A2.01/868T and A2.01/868C cells using luminex method. A2.01, A2.01/868T and A2.01/868C cells were either stimulated by staining with anti-CD4 (SIM4) or incubated with media (unstimulated) for 45 minutes in ice and then cross-linked with goat anti-mouse monoclonal antibodies for 3 minutes at 37°C. The reaction was stopped by adding lysing solution. Each sample was aliquoted into six wells and stained with labeled with fluorescent conjugated anti-phosphotyrosine (4G10) and analyzed using Luminex system. The mean fluorescence was determined for each sample. Stimulated A2.01/868T cells had significantly higher amounts of phosphorylated p56lk compared to stimulated A2.01/868C cells. This experiment was repeated three times with similar results. The error bars represent ± SE.
Phosphorylation of Tyr505

Figure 21: Determination of the amount of phosphorylated tyrosine residues 505(tyr505) residue on Lck between A2.01/868T and A2.01/868C cells. A2.01/868T and A2.01/868C cells were either stimulated by staining with anti-CD4 (SIM4) or incubated with media (unstimulated) and cross-linked with goat anti-mouse monoclonal antibodies. The cells were stained for phosphorylated p56Lck at residue tyr505 by intracellular staining using anti-tyr505 PE and analyzed using flow cytometer. Phosphorylation of p56Lck at tyr505 is higher in the A2.01/868T cells compared with A2.01/868C cells. These results represent same experiment done five different times. Significantly higher amounts of phospho-tyr505 (p=0.02) were detected in CD4 868T cells than in CD4 868C cells.
5.4.2 Evaluating the impact of CD4 signaling on A2.01/868T cell immune activation status.

Rationale

Epidemiological studies done on the sex workers cohort in Nairobi, Kenya, have shown that having CD4 868T is associated with a more rapid loss of CD4+T cells among HIV-1 infected individuals (Francois Vouriot, thesis 2005). One of the possible mechanisms for this rapid T cell loss could be activation-induced cell death (AICD). AICD is a mechanism by which activated cells die by apoptosis or programmed cell death upon receipt of signals through T cell receptor (TCR) and co-receptors such as CD4 (Newell, MK, 1990). The signals induced through CD4 have been shown to play a major role in the activation of T cell. Cross-linking of CD4 with gp120 or anti-CD4 monoclonal antibodies known to be specific for HIV-gp120 binding site has been shown to result in a rapid phosphorylation of p56\textsuperscript{lck} activity (Phipps, DJ, 1996, Juszczak, RT, 1991). Phosphorylation of p56\textsuperscript{lck} at tyr394 of lck leads to AICD which is a direct downstream functional effect of activation of the lck signaling pathway. Since AICD has been proposed to play a significant role CD4+T cell depletion, we sought to investigate whether it is the main mechanism for CD4 T cell loss in HIV infected persons with CD4 868T allele.

Hypothesis

The rapid T cell loss seen in HIV infected subjects with CD4 868T allele could be due enhanced sensitivity of HIV-1 uninfected CD4+T cells to antigen enhanced activation-induced cell death. The binding of gp120 to CD4 Trp240 cells initiates a cascade of events that lead to elevated immune activation status. Activated cells are likely to proliferate and die due to apoptosis. It is
for these reasons that we hypothesized that the rapid loss of CD4+T cells in persons with CD4 868T allele is due to activation-induced cell death.

Objectives:

1. To investigate if stimulation of PBMCs by cross-linking with anti-CD4/anti-CD3 results in differences in immune activation status between CD4 868T/C and CD4 868C cells.

2. To investigate if there are differences in the rate of apoptotic cell death between stimulated A2.01/868T and A2.01/868C cells.

Study outline

PBMCs obtained from subjects with either CD4 868C/T (heterozygous) or CD4 868CC (homozygous wild-type) were isolated as described in the methods section. As the CD4 868TT are rare we were unable to find any to study. Cells were either stimulated using anti-CD4/Goat anti-mouse/anti-CD3. After six hours of stimulation, cells were stained with anti-CD4, HLA-DR and CD69 to look for the elevation of any of the immune activation markers on the CD4+T cells. Similarly, a separate set of cell line cells were stimulated and stained with annexin-V to determine the percentage of cell death due to apoptosis.

Results

A total of 15 donors consisting of 8 donors with CD4 868CC and 7 with CD4 868T/C alleles were used in the study. The PBMCs were stimulated with anti-CD4/anti-CD3 as described above and analyzed for both HLA DR and CD69 expression. A comparison of the unstimulated and stimulated PBMCs from the two groups of donors showed that a mean of 17% and 22% of CD4
868T/C cells were positive for HLA DR from an un-stimulated and stimulated cells, respectively. The difference between these means was statistically significant (Figure 22B, p=0.034). On the other hand, mean percentages of 15% and 17% were observed from unstimulated and stimulated CD4 868CC cells, respectively, and the differences in the means were not statistically significant (Figure 22A, p=0.719). These results suggest that exposure of CD4 868T/C cells to HIV-1 envelope may lead to a higher activation state compared to exposure of the same antigen to CD4 868CC cells.

In addition to using HLA DR, another immune activation marker, CD69 was used to compare immune activation levels between the unstimulated and stimulated cells in the two groups of donors. Analysis of stimulated PBMCs from the two groups of donors revealed that 30% and 23% of CD4 868T/C and CD4 868C were positive for CD69 respectively. Although CD4 868T/C group had a higher mean percentage of CD69 cells compared to CD4 868CC cells, the differences in the mean were not statistical significant (Figure 23, p=0.207). CD69 is an early immune activation marker and both cells with either CD4 isoforms showed a strong activation following cross-linking. These results suggest that CD4+T cells from individuals with CD4 868T when exposed to gp120, may become activated, according to the HLA DR results.

One of the proposed mechanisms for rapid CD4+T cell loss in HIV-1 infected persons with CD4 868T allele is apoptosis. In order to investigate the role of apoptosis in rapid loss CD4 cells in these persons, A2.01/868T and A2.01/868C cells were stimulated by cross-linking using both anti-CD4 and anti-CD3. After an overnight incubation, cells were stained with annexin-V PE and analyzed for apoptosis. Results from this experiment showed that 35% of A2.01/868C cells
positive for annexin-V compared to 50% from A2.01/868T cells. These results suggest that exposure of A2.01/868T cells to gp120 may induce rapid cell death by apoptosis compared to A2.01/868C cells (Figure 24).
Figure 22: Comparing HLA-DR expression levels between un-stimulated and stimulated CD4 868T/C and CD4 868C cells. PBMCs were obtained from 8 individuals with either CD4 868CC or 7 individuals with CD4 868C/T. The cells were either stimulated with anti-CD4/Anti-CD3 or were un-stimulated and incubated for 2 hours at 37°C. The un-stimulated and stimulated cells were stained for the expression of HLA DR. The mean percentages of CD4 868CC positive cells for HLA DR were 15 and 17 for unstimulated and stimulated, respectively. No statistical significance was observed between the two groups (p=0.719) and between 868CC unstimulated and stimulated (p=0.719). The mean percentage of CD4 868T/C cells positive for HLA DR were 17 and 22 for unstimulated and stimulated, respectively. The difference between the means were statistically significant (p=0.034). The error bars represent SEM.
Figure 23: Comparing CD69 expression on A2.01 based cell lines and PBMCs from CD4 868T/C and CD4 868CC cells. PBMCs were obtained from 15 blood donors. The donors were divided into two groups of 7 donors with CD4 868T/C and 8 with CD4 868CC genotypes. Either A2.01 based cell lines (A) or PBMCs (B) were stimulated with anti-CD4 or culture in media alone as un-stimulated, incubated for 2 hours at 37°C, stained for the expression of CD69 and analyzed by flow cytometer. (A) A significantly higher percentage of CD69 expressing cells were detected from A2.01/868T cells than from A2.01/868C cells (p=0.03). (B) A mean of 30% of stimulated PBMC with the CD4 868T/C genotype were CD69 positive compared to a mean of 23% of stimulated CD4 868CC cells. A comparison of the means of stimulated CD4 868T/C and CD4 868CC cells revealed no statistical difference between the two groups (p=0.207).
Figure 24: Comparing the rate of apoptotic cell death activated A2.01/868T and A2.01/868C cells. A2.01 cells and cells expressing similar levels of either CD4 isoform were stimulated by staining with anti-CD4 and incubated overnight at 37°C. All the cells were stained with annexin-V PE and analyzed by flow cytometry. The results from flow cytometry show that (A) significantly higher (p=0.005) numbers of stimulated A2.01/868T cells were undergoing cell death than A2.01/868C cells. (B) Similarly A2.01/868T cells had a higher percentage (50%) of cells staining for annexin-V compared to 35% of A2.01/868C cells staining positive for Annexin-V.
6.0 Discussion

Although HIV/AIDS has been recognised for almost three decades, the virus continues to spread in different populations of the world, with sub-Saharan Africa carrying the heaviest burden. Transmission of HIV from an infected to uninfected individual and the outcome of infection are dependent on a number of factors including host genetics. Host genetic variations, particularly those directly involved in HIV-1 cell entry have been shown to profoundly modify individual responses to HIV-1 exposure, infection and pathogenesis. In particular, genetic variations associated with the HIV-1 co-receptors, CCR5, CXCR4 and their ligands have been documented (Gonzalez, E, 2001, Martin, MP, 1998, Smith, MW, 1997).

Even though it has been known for decades that there are genetic variations in the gene encoding the CD4 molecule and that CD4 is the primary receptor for HIV-1, there are no studies that have looked at the association between these CD4 variants and risks to HIV-1 infection and pathogenesis. However, a recent epidemiological study from our laboratory reported in the rationale section of this thesis found some important associations between the CD4 868T allele and risks of HIV-1 infection. In brief, these studies found that having the CD4 868T allele was associated with increased susceptibility to HIV infection as well as rapid disease progression. Although these findings were intriguing, they also left a number of unanswered questions. For example, what was the molecular basis for this association? Could the mutant CD4 bind gp120 with higher affinity, hence causing cells to be more readily infected? Or could the mutant CD4 have changed in a way that its interaction with gp120 makes these cells hyperactivated and therefore undergo programmed cell death or apoptosis? The role played by activation induced cell death in CD4+T cell loss in HIV-1 infected persons is well known. Infections of both human
and primates with their respective lentiviruses, result in different disease pathways depending on whether activation induced cell death is present or not. In humans, infection with HIV-1 is characterized by abnormally high levels of lymphocyte apoptosis in peripheral blood and lymph nodes (LN) leading to AIDS (Laurent-Crawford, AG, 1991, Lewis, D. E., 1994). On the other hand, infection of Sooty Mangabes with SIV where activation induced cell death is a rare occurrence does not lead to AIDS. Although evidence from the literature show activation induced cell death as one of the major contributors to CD4+T cell loss, its role in the rapid loss of CD4+T cells in people with CD4 868T allele is unknown. It was these questions that led us to investigate the exact molecular basis for the associations of CD4 868T and the risks of HIV-1 infection as well as disease progression.

6.1 CD4 polymorphism and HIV disease association

Although the CD4 molecule was assumed to be non-polymorphic, there are a number of documented synonymous polymorphisms in the CD4 gene. These include polymorphisms in the region of the transcription start site on the CD4 gene (Edwards MC, 1991), in an intron region on CD4 gene (Edwards MC, 1992) and on the promoter region of the CD4 gene (Kristiansen OP, 2004). In 1981, Bach et al identified the first non-synonymous polymorphism on the coding region of the CD4 gene which resulted in the loss of the OKT4 epitope (Bach MA, 1981). Subsequent studies revealed that the genetic basis for OKT4 epitope deficiency was due to a SNP at the position 868 (C → T) of the CD4 gene. This nucleotide change resulted in a single amino acid substitution of tryptophan for arginine at amino acid residue 240(CD4 Trp240) in the third domain (D3) of the extracellular portion of the CD4 molecule (Hodge TW, 1991, Lederman S, 1991).
As mentioned in the rationale section of this thesis, the CD4 868T has a high prevalence among Africans and the lowest prevalence in Caucasian populations (Fuller TC, 1984). The fact that this polymorphism is found in high prevalence among people of African descent and that the highest HIV/AIDS prevalence is also found in the same population, prompted our group to conduct epidemiological studies to investigate whether CD4 868T was associated with any increased risk of HIV infection and disease progression. A cohort of HIV high risk commercial sex workers (csw) and another cohort of HIV low risk (non-csw) women from the Pumwani district of Nairobi, Kenya, were used for these studies. A Masters student in Dr. Fowke's laboratory, Fracoise Vouriot, used amplification refractory mutation system polymerase chain reaction (ARMS-PCR) as a screening method and a sequence based assay to confirm CD4 sequence on DNA samples from 364 high risk commercial sex workers, 284 mothers and 276 children. Analysis of the influence of CD4 polymorphism on HIV incidence revealed that individuals with CD4 868T allele had a shorter time to seroconversion compared to individuals with CD4 868C allele (Figure 7). Further analysis revealed that HIV low-risk mothers who had the CD4 868T allele were about 3 times more likely to be rapid progressors compared to those CD4 868C allele (Figure 8). Additionally, mothers carrying the CD4 868T allele had a 19-fold higher risk of transmission of HIV to their babies through breast feeding than mothers who did not have the allele (Vouriot, F, Thesis. 2005). Prior to our study, a number of studies had been done to look for associations between CD4 868T and disease. Among these diseases are; Systemic Lupus Erythematosus (SLE) (Stohl W, 1985), Graves' disease (Fukuda T, 1984), and between patients with and without thymoma, hypogammaglobulinemia, and red cell aplasia (Levinson Al, 1985). In all these studies, similar frequencies of CD4 868T were found in subjects with and without the
disease. The results of these studies suggest that CD4 868T had no impact on predisposing these individuals to developing the diseases mentioned above.

As mentioned above, our group’s finding that CD4 868T is associated with increased susceptibility to HIV-1 infection and rapid disease progression was the first time a polymorphism of CD4 was associated with an increased risk of a disease. However, this was not the first time a polymorphism of a receptor protein had been reported to be associated with human disease. There are several reports of infectious diseases in which a single gene polymorphism has altered the host susceptibility to infection as well as the course of the disease. Such examples include tuberculosis (Bellamy R, 1998), leprosy (Meisner, SJ, 2001), Helicobacter pylori infection (Malaty HM, 1994), and Hepatitis B virus persistence (Lin TM, 1989). Another example is from a study of the relationship between the Duffy blood group antigen and susceptibility to Plasmodium vivax malaria. The malaria parasite Plasmodium vivax invades human erythrocytes by binding to Duffy antigen/chemokine receptor (DARC) expressed on the erythrocyte surface. Individuals who have a single nucleotide polymorphism in the DARC promoter region have a suppressed DARC expression in erythrocytes and are completely protected against infection with P. vivax, but not against other species of malaria parasite, which invade erythrocytes through different receptors (Miller LH, 1976, Tournamille C, 1995). In all these cases, the genetic variations in the host either have altered the function of the protein or change its expression, thereby, making the host either more resistant or more susceptible to infection with a particular infectious agent.
In HIV infection too, a number of genetic variations in the co-receptor genes that alter individuals susceptibility to infection and disease progression have been reported. CCR5, one of the co-receptor for HIV has several natural SNPs within the coding and regulatory regions of its gene, which appear to have an impact on HIV-1–related pathogenesis. In its regulatory region, CCR5 has a G→A polymorphism at nucleotide position (np) 590299 (Martin MP, 1998), a T→C polymorphism at np 59353, and a C→T polymorphism at np 59356 (Kostrikis LG, 1999), which have been reported to be involved in determining the rate of progression to AIDS. Additionally, some of polymorphisms in the promoter region of CCR5 have been found to increase the risk of perinatal transmission of HIV-1(Kostrikis LG, 1999). CCR2, a minor co-receptor for HIV-1, has a polymorphism at np 180 (G→A polymorphism) in the coding region of its gene that has been associated with slower disease progression in HIV-1–infected adults (Kostrikis LG, 1998, Smith MW, 1997). In all these instances, the molecular basis of the SNP association with susceptibility to HIV infection and disease progression has not been well defined although it is suspected that any of these non-synonymous SNP could alter the expression CCR5. Thus genetic heterogeneity to infection susceptibility observed between individuals is not confined to only these diseases, but also includes diseases such as AIDS.

Since non-synonymous SNPs cause amino acid changes in the sequence of the receptor protein, it is generally agreed that the mechanisms by which they might alter susceptibility to infection is by either interfering with host cell receptor expression protein or binding affinity to ligand. To date, the only reported mechanisms for altered susceptibility to infections due to genetic variations in the receptor genes are those that result in low expression, no expression or expression of a truncated form of the protein (Sunyaev, S, 2001).
As reported elsewhere (Figure 9B) in this thesis, CD4 868T does not cause CD4 to be expressed in lower levels or a truncated form. Indeed, staining of CD4+ T cells with anti-Leu-3a that binds to a conserved CD4 epitope, found no differences in CD4 expression levels on cells from subjects with polymorphic CD4 and those with wild type CD4 (Takenaka T, 1993). These findings suggest that the risk of HIV infection and disease progression related to the CD4 polymorphism is not due to receptor density. This led us to speculate on other possible mechanisms whereby CD4 SNP could affect risk to HIV infection and disease progression. In theory, these proposed mechanisms may or may not hold true until proven by well designed in vitro and in vivo studies. Likewise, the epidemiological studies that reported the association between CD4 868T and risks to HIV infection and disease progression must be confirmed by well designed in vitro studies.

6.2 Mechanisms of CD4 868T on the risks to HIV infection and disease progression.

The epidemiological findings reported by our group clearly demonstrated that individuals who have the CD4 868T genotype are more susceptible to HIV infection and upon infection progress faster to AIDS compared to those with the wild-type CD4 genotype. Knowing that the CD4 polymorphism does not alter the CD4 expression and that it does not occur where gp120 of HIV binds, a possible mechanism for susceptibility to HIV infection would be that CD4-Trp240 binds gp120 with higher affinity and allowing cells to be more readily infected. If this is true then the enhanced binding affinity between CD4 Trp240 and gp120 may have other consequences on the function of the CD4 molecule. The most important functions of the CD4 molecule are those as a receptor to HIV virus and a signal transduction receptor. As such, the polymorphism may alter
either or both functions of CD4. Secondly, a possible mechanism for rapid disease progression in individuals with CD4 868T, is that signals transduced by gp120 binding to CD4 Trp240 result in rapid activation of these cells. Having more activated cells would provide more targets for HIV infection and at the same time could lead to rapid loss of these cells through activation induced cell death. For the purposes of investigating the association between the CD4 868T and the increased susceptibility to HIV infection and disease progression, a CD4-lymphoblastoid T-cell line, A2.01 was chosen.

6.3 A2.01 cell line, a model for studying the impact of CD4 SNP on the functions of CD4.

As described in the method section, the A2.01 cell line, a CD4-lymphoblastoid T-cell line derived from the CD4+T cell line A3.01, was transfected with CD4 868C and CD4 868T genes. The A2.01/868T and A2.01/868C cells expressing similar levels of CD4 (figure 9) were selected and used for all functional assays. Although PBMCs from humans could have been ideal for this study, the A2.01 cell line was chosen for several reasons.

First, A2.01 cell lines were chosen for their suitability for HIV infection studies compared to PBMCs. Although PBMCs from individuals with CD4 868T and CD4 868C could have been used for this study, these cells have a number of disadvantages. One, susceptibility to HIV infection varies in cells obtained from different donors due to multiple parameters (Paxton WA, 1996). Two, PBMCs require phytohemagglutinin (PHA) stimulation before HIV infection and inclusion of interleukin-2 to maintain cells during the culture period. Both of these factors produce an in vitro infection environment that differs from that in vivo. On the other hand, A2.01 cells, unlike, PBMCs; need no activation, no conditioned medium and no Interleukin-2 in order
to show infectivity. Therefore, the environment in which HIV infection of A2.01 cells *in vitro* occurs is similar to that *in vivo*.

Third, the A2.01 cell line unlike PBMCs is easy to manipulate in a way that makes it possible to select for cells expressing similar levels of CD4. PBMCs from different donors are likely to have different levels of CD4 expression on their CD4+T cells. CD4 expression is one of the most important determinants of susceptibility to HIV-1 infection. Cells with low expression of a receptor are less susceptible to infection whereas cells which express high levels of receptor proteins are likely to have an enhanced susceptibility to infection. The inability to control for CD4 expression in PBMCs would have introduced additional confounding factors that would made the interpretation of the results difficult.

Fourth, because A2.01 cell line is a CD4+T cell derivative, it was assumed that it has similar host proteins as CD4+T cell apart from CD4 expression. HIV-1, like other lentiviruses, requires a number of host proteins for its replication (Greene WC, 2002). Due to the similarities between A2.01 cell lines and CD4+T cells, it was assumed that it would provide all the host proteins that are required for HIV-1 replication.

Like most cell lines, A2.01 cells, when propagated for a long time, undergo some changes. Since HIV-1 is dependent on host proteins for its replication, any changes within either A2.01/868T or A2.01/868C cells could have a significant impact on our results. One such change could have been the alteration of CD4 expression on either A2.01/868T or A2.01/868C cells. To strictly control for this possibility, CD4 expression on A2.01/868T and A2.01/868C cells was monitored.
prior to every experiment. Due to their suitability as an *in vitro* model for studying the molecular basis for CD4 868T effects on HIV infection, A2.01 and its derivatives were used for investigating; i) if CD4 868T cells are more susceptible to HIV infection and (ii) if CD4 Trp240-specific signal transduction leads to rapid activation and apoptotic cell death of the CD4 868T cells

**6.4 CD4 868T cells are more susceptible to HIV infection than CD4 868C cells.**

Susceptibility to HIV infection is determined by many factors including genetic variations in the receptor proteins that play a major role in individual infectiousness (Kaslow, RA, 2005). To determine if the epidemiological indications that 868T results in increased susceptibility to HIV-1 could be mimicked *in vitro*, HIV-1 infection studies were performed. Our hypothesis was that A2.01/868T cells have increased susceptibility to HIV-1 and therefore are more easily infected with HIV-1 compared to A2.01/868C cells. Results from the infection studies using a laboratory adapted virus HIV-1\textsubscript{MDM} and a Kenyan primary HIV- isolate ML 1956 showed that exposure of the cells expressing different CD4 isoforms to these viruses resulted in more HIV-1 p24 from A2.01/868T cells compared to A2.01/868C cells (Figures 12 and 14). The enhanced p24 levels in supernatant from 868T cells are suggestive of increased susceptibility of these cells to HIV-1 infection. There are three possible reasons for the increase in susceptibility of A2.01/868T to HIV-1 infection. The first possibility is that the increased susceptibility of CD4 868T cells to HIV infection is due to one of the host factors that regulate HIV replication (see next paragraph). The second possibility is that CD4 Trp240 bind to HIV-1 with higher affinity thereby allowing HIV to infect CD4 868T cells more easily (see section 6.5).
The third possibility for the increased susceptibility of CD4 868T cells to HIV infection is that CD4 Trp240-specific signal transduction may enhance HIV replication. A candidate protein for this function is the inactive form of p56lck. For the molecular basis of how p56lck can regulate HIV replication, see section 6.7.

Since the HIV-1 virus has a simple genetic make-up, it requires many cellular factors in order to complete most of its replication cycle. A recent paper in Science identified more than 250 host proteins that regulate viral replication (Brass, AL, 2008). Although many of these host factors favor viral replication, a few of them can restrict viral replication. Such factors include APOBEC3G, TRIM5α and Titherine. APOBEC3G, a human cytidine deaminase family of enzymes restrict HIV replication by deaminating cytosine residues to uracil in the growing minus strand viral DNA during retroviral reverse transcription causing genome degradation or hypermutations (Mangeat B, 2003). TRIM5α like APOBEC3G, acts early during the viral replication by binding to an incoming viral particles to its C-terminal PRY/SPRY domain and rapidly recruits them to the proteasomal degradation (Huthoff H, 2008). Other factors, such as receptor proteins and cyclin T, which are important for viral entry and regulation of viral gene expression, respectively (Sheehy AM, 2002) may be mutated in either A2.01/868T or A2.01/868C cell lines. Any changes in the host cell proteins in these cell lines may alter the susceptibility of these cells to HIV-1 infection. While the infection experiments reported above were very encouraging, we wanted to investigate whether a host factor required for HIV replication was responsible for the increased susceptibility to HIV infection. We repeated HIV infection experiments using a pseudotype HIV-1 virus that infect CD4+T cells in a CD4-independent manner. This HIV-1 virus differs from the viruses used in the previous HIV-1
infection experiments in that its gp120 is replaced with Vesicular Stomatitis Virus (VSV) membrane glycoprotein and its nef gene is replaced by a luciferase gene. Exposure of A2.01/868T and A2.01/868C cells to this virus revealed that both CD4 isoforms had similar levels of susceptibility to pseudotyped HIV-1 infection (Figure 15). This demonstrates that the A2.01 derivatives are equally capable of supporting HIV-1 replication. This control experiment confirms that the differences in infection with the normal HIV-1 virus (HIV-1 HXB) were due to the CD4 isoforms and not differences in the ability to support HIV replication. Since the p24 ELISA used in these studies measured a product (p24 antigen) of HIV-1 replication, it was difficult to judge whether the differences in p24 antigen between A2.01/868T and A2.01/868C cells were due to CD4 and not due to events that determine susceptibility to infection or by other factors. However, it is possible that once exposed to HIV-1 gp120, 868T cells are more activated and it is known that HIV-1 infects and replicates in activated cells better than resting cells. HIV-1 replication is a complex process and the outcome of replication is dependent on factors associated with the virus entry, fusion, transcription, and integration, among others. Any of these factors can influence the rate of viral replication and determine the amount of p24 produced by the infected cells. The infection studies reported here has recently been accepted for publication in The Journal of Infectious Diseases. While we may not know which of these steps influenced HIV-1 replication in either of the CD4 isoforms, it was clear that exposure of these cells to HIV-1 results in a more productive infection of the CD4 868T cells than in CD4 868C cells. A major suspected mechanism for increased susceptibility of CD4 868T cells to HIV infection is the enhanced binding affinity between gp120 and CD4 Trp240.
6.5 A higher CD4 binding affinity to gp120 is not a mechanism for increased susceptibility to HIV infection in individuals with the CD4 868T allele.

The two most important factors that determine the efficiency of HIV-1 entry into CD4+T cells are receptor density and binding affinity. However, we know from previous experiments (Figure 9B) that the CD4 868T has not altered the receptor density of CD4 molecules on T cells, leaving binding affinity as a possible mechanism for affecting the efficiency of HIV-1 entry. In HIV-1 infection, the initial events leading to HIV-1 entry into a CD4+T cells involves the attachment of HIV envelope glycoprotein gp120 to its cellular receptor CD4 followed by attachment to a chemokine receptor (CXCR4 and CCR5). The high binding affinity between the wild-type CD4 and gp120 has been found to be critical for the induction of conformational changes in HIV-1 envelope glycoprotein that promote the membrane fusion process (Kwong, PD, 1998). It is based on this that we hypothesize that enhanced binding affinity between CD4-Trp240(868T) and gp120 of HIV is a mechanism for increased susceptibility to HIV-1 in persons with CD4 868T allele. In order to investigate whether gp120 binds to the CD4 isoforms with different affinities, A2.01/868T and A2.01/868C cells expressing similar levels of CD4 were incubated with gp120 as described in the methods section. Both supernatant and cells were harvested and used to measure the amount of bound gp120 using ELISA and flow cytometry, respectively. There are many commercial binding affinity assays currently available including BIACORE (Biacore AB, Uppsala, SW) and Kinexa (Kinetic Exclusion Assays) (Sapidyne Instruments, Inc., Boise, ID). Most of these assays require the use of soluble proteins. Since we wanted to investigate the binding affinity of gp120 to cell surface bound CD4 (CD4 in its native form), we chose to use ELISA and flow cytometry, both of which allow us to measure binding of gp120 to a native CD4 molecule. Analysis of both ELISA (Figure 17) and flow cytometry (Figure 18)
results revealed that gp120 had a higher binding affinity to CD4 868C cells than CD4 868T cells. These results contradicted our hypothesis and, therefore, suggested that stronger binding affinity between gp120 and CD4-Trp240(868T) is not the mechanism for increased susceptibility to HIV-1 infection in individuals with CD4 868T allele.

To fully understand the association between the low binding affinity between gp120 and mutant CD4 with susceptibility to HIV-1 infection, it is important to take a close at look at the events that lead to viral fusion and entry into a host cell. The first step in HIV-1 replication involves gp120 binding to CD4. This interaction leads to conformational changes in gp120, which further lead to the exposure of the high-affinity co-receptor binding site (Rizzuto CD, 1998). The form gp120 takes after its interaction with CD4 depends on the binding affinities between the two molecules. A lower binding affinity between gp120 and CD4-Trp240(868T) may lead to a different conformation of gp120 which may bind to a chemokine receptor with different affinities. Since, chemokine receptor binding to gp120 also triggers additional conformational changes in the envelope glycoprotein complex that ultimately leads to the fusion of the viral and target cell membrane, this binding may be an important determinant of susceptibility to HIV-1 infection. Overall, susceptibility to HIV-1 infection at the cellular level depends on the binding affinities between gp120 and CD4 and between gp120 and a chemokine receptor. These findings together with the epidemiological findings led us to speculate on two possible relationships between the observed low binding affinity of CD4 Trp240 for gp120 and increased susceptibility to HIV-1 infection.
The first possibility is that HIV entry into cells expressing either wild-type CD4 or CD4 Trp240 is not limited by the differences in the receptor binding affinities to gp120. While it is a known fact that viruses which have higher affinity for receptor proteins are more efficient in initiating cellular entry than those with low affinity (Moore, JP, 1993), some in vitro studies suggest that the relationship between high and low affinity of a ligand to a receptor is dependent on the receptor density. At higher receptor density, viruses with low or higher affinities to their receptors have been shown to cause similar infection rate (Adams GP, 2006). Several studies, while looking at the relationship between gp120-CD4 binding affinity and CD4 receptor density on infectivity of various cells, found that viruses with low affinity to CD4 had decreased infectivity in cells with low CD4 expression such as H9 lymphocytes. However, equal HIV infectivity was observed in cells with high CD4 expression such as supT1 lymphocytes, when viruses with either low or high binding affinity to CD4 were used (Thali M, 1991, Moore, J, 1992, Kabat, D, 1994). These studies, therefore, suggest that the differences in binding affinity alone may not play a rate limiting role in determining susceptibility to infection as long as the receptor density is above a certain threshold. In the case of the CD4 polymorphism, we have shown previously that the receptor density has not changed in CD4 868T cells, suggesting that the differences in binding affinity between gp120 and the two CD4 isoforms may not limit susceptibility to HIV-1 infection.

The second possible explanation for the increased susceptibility of CD4 868T cells to HIV infection is that the low binding affinity between gp120 and CD4 Trp240 enhances gp120/CXCR4 binding affinity. For the efficient entry of HIV virus into a cell, the binding affinity between gp120 and CD4 alone may not be the sole determinant of susceptibility to HIV-
1 infection. Indeed, previous studies have shown that expression of human CD4 on murine T cells does not render these cells permissive to HIV-1 infection suggesting that in addition to CD4, a chemokine receptor such as CCR5 or CXCR4 is an important determinant of susceptibility to HIV-1 infection (Alkhatib, G, 1996, Deng, H, 1996). Furthermore, as was mentioned previously, individuals harboring a homozygous 32 base pair deletion in the CCR5 gene, despite having adequate CD4 on their T cells, are resistant to HIV-1 infection, further suggesting that the interactions between gp120 and a chemokine receptor also play a significant role in determining susceptibility to HIV-1 infection (Liu R, 1996, Huang Y, 1996). Since HIV-1 binding to CD4 leads to a conformational change in gp120, we can speculate that the susceptibility to HIV-1 infection is dependent on the form gp120 takes after its interaction with the CD4 molecule. The conformational change in the gp120 can (a) expose an epitope on gp120 with enhanced binding affinity to either CCR5 or CXCR4 or (b) lead to enhanced exposure of the V3 loop of gp120 (Rizzuto CD, 1998). Either of these mechanisms can lead to rapid fusion of the virus envelope to the cell membrane, thereby facilitating virus entry.

While chemokine receptors such as CCR5 have been shown to bind gp120 with low affinity in the absence of CD4, this binding is greatly enhanced if gp120 first binds to CD4 before interacting with a chemokine receptor (Lijun Wu, 1996), suggesting that binding affinity between gp120 and CD4 could play an important role in the subsequent events leading to HIV-1 entry. In particular, the binding affinity between gp120 and CD4 molecule could determine the binding affinity between the virus envelope protein and a chemokine receptor. The correlations between the gp120-CD4 molecule and gp120-chemokine receptor binding affinities have not been fully investigated. While binding between gp120 of HIV and CD4 molecules is important
for HIV virus entry into a CD4+T cell, this interaction can also induce signals that lead to activation of the cell and subsequent cell death.

6.6 AICD is a mechanism for rapid disease progression in persons CD4 868T allele.

As mentioned in the previous section, in addition to binding gp120 to help the HIV virus gain entry into a host cell, CD4 has been shown to play an important role in activation of T cells (Konig R, 1995). Indeed, it is now accepted that gp120 can activate T cells through its interaction with CD4, although there have been some controversies between different studies (Benkirane M, 1994, Briant L, 1996, Chirmule N, 1994). Activation of CD4+ T cells whether through gp120 binding to CD4 or through CD4 binding to major histocompatibility (MHC) II on antigen presenting cells, leads to expansion and then contraction of the cell populations. The contraction phase of this process is characterized by programmed cell death or apoptosis. Because this kind of cell death is initiated by activation signals, it has been named activation induced cell death (AICD). As was mentioned in the introduction section of this thesis, HIV-1 infection leads to the gradual loss of CD4+T cells that culminate in immunodeficiency. Several mechanisms have been suggested to explain the molecular basis of immunodeficiency. They include; 1) HIV mediated cell killing (cytopathic death), 2) Syncytia formation or fusion of infected and uninfected CD4+T cells to form multinucleated giant cells, 3) Antibody-dependent cellular cytotoxicity (ADCC), 4) cytotoxic T lymphocyte (CTL) killing of infected CD4+T cells, and 5) activation-induced cell death (AICD). Of these mechanisms, AICD seems to be the most relevant for rapid CD4+T cell loss in individuals with CD4 868T allele for the following reasons. First, previous studies have shown that the numbers of productively infected CD4+T cells are too low to account for all the CD4+T cell loss occurring during HIV infection (Chun TW, 1997).
Secondly, the numbers of CD4+ T cells undergoing apoptosis has also been shown to be much higher in uninfected than in productively infected CD4+ T cells (Embretson J, 1993). These studies suggest that the majority of the CD4+ T cells lost in HIV infected individuals are due to apoptosis of uninfected or bystander CD4+ T cells. Indeed, HIV-1 induced apoptosis in bystander cells has been implicated as the major mechanism of the depletion of T cells (Phenix BN, 2003, Debatin KM, 1994). Although AICD has been proposed to be the main cause of bystander CD4+ T cell death, the exact mechanisms have not been fully elucidated. Currently, there are two proposed mechanisms for the death of bystander CD4+ T cells, namely; HIV proteins released from infected cells acting on neighboring uninfected cells, or bystander cells die due to activation-induced cell death (AICD).

The HIV-1 proteins that have been shown to be the cause of bystander cells death include; gp120, Tat, Vpr, and Nef (Cao J, 1996, Geleziunas R, 2001, Stewart SA, 2000). Of interest to this thesis is the role of envelope protein gp120 on HIV pathogenesis. In HIV infected individuals, gp120 is found to be cell-associated, virus-associated or free gp120. The mechanisms underlying the role played by the interaction of gp120/CD4 in inducing death of bystander CD4+ T cells will be discussed later. Because, AICD is proposed to be a mechanism in the rapid CD4+ T cell loss in CD4 868T individuals, the events leading to bystander cell death were investigated. These events include, 1) phosphorylation of lck, 2) elevation of immune activation markers on stimulated CD4+ T cells and 3) levels of apoptosis between CD4 868T and CD4 868C cells. Each of these mechanisms will be discussed in details in the subsequent sections.
6.7. gp120-induced signal transduction enhances HIV-1 replication in CD4 868T cells.

In addition to its role as a receptor for HIV-1, CD4 also plays a role as a signal transduction receptor. CD4 achieves this function through its interaction with a protein tyrosine kinase, p56\textsuperscript{\textit{lk}} (lck). Coupled to CD4 through an interaction between the cytoplasmic tail of CD4 and the NH2-terminus of lck, p56\textsuperscript{\textit{lk}} participates in T cell activation by tyrosine phosphorylation of downstream proteins involved in the activation pathway (Straus DB, 1992, Veillette A, 1988). Indeed, it has been demonstrated that p56\textsuperscript{\textit{lk}} linked to CD4 is critical for antigen-dependent T-cell activation (Veillette A, 1988). Furthermore, the same studies demonstrated that cells expressing mutant forms of p56\textsuperscript{\textit{lk}} lacking the kinase activity were unable to become activated following antigenic challenge (Abraham N, 1991).

In HIV-1 infected persons where gp120 is found in different forms, either the virus-associated or soluble forms of gp120 can act as a priming molecule. Therefore, the interaction between gp120 and CD4 molecules in these patients may lead to activation of either HIV-1 infected or HIV-1 uninfected CD4+T cells. Additionally, it has been shown that HIV-1 can take advantage of the signal transduction of CD4 to modulate components of the intracellular virus life cycle such as viral replication and apoptosis to its advantage (Laurence B, 1996). Since our epidemiological studies had shown that having CD4 868T is associated with rapid decline in CD4+T cells, we investigated the role of gp120-induced signal transduction on the outcome of HIV-1 infection.

We hypothesized that gp120-induced signal transduction enhances viral replication by phosphorylation of tyr505 of lck. In this study, A2.01/868T and A2.01/868C cells were cross-linked with anti-CD4 that mimics gp120 binding to CD4. Cross-linking of CD4 has been used extensively in many studies including ours to study the signaling events through CD4 molecule
According to these studies, cross-linking of CD4 using anti-CD4, mimics the aggregation of CD4 molecules induced by the interaction of CD4 with major histocompatibility complex class II molecules on antigen presenting cells (Luo Kunxin, 1990).

As mentioned earlier, the interaction between gp120 and CD4 can generate signals that can either lead to activation of T cells or signals that may enhance viral replication. The ability of these signals to either cause T cell activation or regulate HIV-1 replication is dependent on the activation state of p56\textsuperscript{ck}. The activity of p56\textsuperscript{ck} is in turn controlled by phosphorylation of two residues on p56\textsuperscript{ck}, namely; Tyr505 and Tyr 394. When phosphorylated at Tyr505, lck attains a conformation that is relatively inactive (Amrein KE, 1988, Marth JD, 1988). On the other hand phosphorylation of Tyr394 leads to activation of lck (figure 6b). In our study, we demonstrated that stimulation of A2.01/868T and A2.01/868C cells by cross-linking with anti-CD4 resulted in more total lck phosphorylation (Figure 19) and more phosphorylation of tyr505 in A2.01/868T cells (Figure 21). This would suggest that exposure of A2.01/868T cells to HIV-1 virus envelope transduces signals that inactivate p56\textsuperscript{ck}. However, we have also shown that 868T/C PBMCs upon cross-linking with anti-CD4 monoclonal antibodies attain a higher activation status than 868CC PBMCs. The question is how do signals that inactivate p56lck activities end up activating the same cells? The answer to this question can be found in the published literature on what is currently known about the regulation of p56\textsuperscript{ck} by CD45, a protein tyrosine phosphatase. Although many studies have reported that hyperphosphorylation of lck at tyr505 would result in less or no activation of the T cells (Cahir McFarland, E. D, 1993, Sieh, M., 1993, Ostergaard, H. L, 1989), there is a lack of consistency between these studies on the influence of this
hyperphosphorylation on lck activity. Some studies have found that lck kinase activity is increased despite hyperphosphorylation of the tyr505 (Burns, CM, 1994, D'Oro, U.1999) and that this was due to hyperphosphorylation of tyr394 (D'Oro, U.1996). Additionally, a study investigating the impact of CD45 on lck activity found that T cell lines that lacked CD45, had lck hyperphosphorylated predominantly on tyr505 and at the same time the lck activity was substantially elevated (Burns, CM, 1994). What all these studies suggest is that the regulation of lck activity by CD45 using tyr505 may be more complex than postulated in the model in Figure 6b. Therefore, lck can become activated either when lck is hyperphosphorylated predominantly on tyr505 or when both tyr505 and tyr394 are hyperphosphorylated. Since we did not measure phosphorylation of tyr394 (anti-tyr394 monoclonal antibody was unavailable commercially), evidence from literature presented here support our conclusion that despite hyperphosphorylation on tyr505, lck activity could be enhanced leading to activation of CD4+T cells in 868T PBMCs. My model for the signaling events in 868T cells is that the initial signals cause hyperphosphorylation of tyr505 which leads to transient inactivation of lck. This is followed by dephosphorylation of tyr505 and hyperphosphorylation of tyr394 which leads to activation of the 868T cells.

The role played by p56\textsuperscript{/lck} in regulating HIV-1 replication has been documented in several studies (Benkirane M, 1994, Benkirane M, 1995, Tremblay M, 1994). These studies have shown that a high initial lck activity prior to HIV-1 infection or sustained elevated of its activity is inversely correlates with productive HIV-1 infection (Tremblay M, 1994, Yousefi S, 2003). Indeed, these studies have shown that cells expressing CD4 having point mutations at cysteines 20 and 23, so that Lck no longer associates with CD4 tails, results in greatly accelerated HIV replication
While this may be one of the factors that could contribute to high viral load in individuals with CD4 868T allele, it may be a minor factor.

The line of argument presented here suggests that individuals with CD4 868T allele when exposed to HIV-1 are likely to attain high viral load with a short disease progression compared to individuals with CD4 868C allele. Although no studies have been done to compare viral load between HIV-1 infected individuals with either CD4 868T or CD4 868C alleles, both the signal transduction and HIV-1 infection assays reported here strongly suggest that individuals with CD4 868T upon infection are likely to have high HIV-1 viral load. High viral load has been shown to be a predictive of the rapidity of disease progression (Michael NL, 1995, Miedema, F, 1988). Overall, we can conclude from these studies that gp120-induced signal transduction is a mechanism for disease progression in CD4 868T individuals infected with HIV virus.
Figure 25: Mechanisms of gp120-induced killing. Soluble gp120 triggers cell death through interactions with CD4. Binding of gp120 to CD4 may stimulate the CD95/CD95L-dependent cell death pathway or trigger a Bax-dependent mitochondrial apoptosis, which requires p56lck activity. In addition, interactions between gp120 and CXCR4 can cause mitochondrial membrane permeabilization (MMP) through pertussis toxin- sensitive G proteins (Gia), p38MAPK pathway and/or Ca2+ -dependent mechanisms.

Permission to use this figure was obtained from Dr. Guido Kroemer. (Perfettin JL, et al, 2005).
6.8 Immune Activation is a mechanism for rapid CD4+T cell loss in CD4 868T individuals.

As mentioned in the previous section, gp120 induced CD4-specific signal transduction can result in phosphorylation of p56^Lck which is the first event in the CD4-lck signaling pathway. The subsequent events in this signaling pathway may or may not lead to T cell activation depending on whether tyr394 residue of lck is phosphosrylated. In HIV-1 infection, having elevated immune activation status has been implicated as a major contributor in the pathogenesis of HIV-1 infection (Hazenberg MD, 2003). Any stimulus that induces elevation of immune activation status in an HIV-1 infected individual is likely to affect HIV-1 pathogenesis. It is based on this background that we hypothesized that activation-induced cell death is a mechanism in rapid loss of CD4+T cells of persons with CD4 868T allele. In order to assess whether immune activation played a role in T cell loss in individuals with CD4 868T/C, A2.01 based cell lines(A2.01, A2.01/868T and A2.01/868C) and PBMCs obtained from individuals with either CD4 868T/C or CD4 868CC genotypes were used. For the A2.01 based cell lines, only the expression of CD69 were analyzed as these cell lines are HLA DR negative, making it unsuitable to measure any changes in immune activation with regard to HLA DR (Folks T, 1985). Stimulation of A2.01 based cell lines revealed that 868T cells attained a higher activation levels than 868C cells(Fig 23A). However, when PBMCs were used similar results were not seen(Fig 23B). In fact, there were not differences in activation levels between the two groups of individuals whether CD69 or HLA-DR were used. The only exception was when the expression of HLA-DR was compared between the unstimulated and stimulated cells between these groups.

We noted a statistical significant increase in HLA-DR in stimulated PBMCs from subjects with CD4 868T allele and not in CD4 868C (Figure22). On the other hand, we noted a significant increase in CD69 on stimulated PBMCs but this increase was similar between the two groups of
study subjects (Figure 23). Described as the earliest immune activation antigen, CD69 can be found expressed on activated T cells within 2–3 hours after stimulation (Santis AG, 1992, Lopez-Cabrera M, 1993). On the hand, HLA-DR, a MHC class II antigen, is expressed on T cells at low levels but expression increases upon antigenic stimulation, reaching a peak after approximately 7 days (Mahalingam M, 1993). Although CD69 could be a good immune activation marker for other infectious diseases (Ferenci K, 2000), studies comparing immune activation levels between HIV-1 uninfected and infected subjects have found no differences in the expression levels of this marker between the two groups (Pitsios C, 2008, Krowka JF, 1996, Nielsen SD, 1998). Since HIV-1 infection is known to cause elevated immune activation, these studies suggest that CD69 could be a good immune activation marker for increasing the susceptibility of a person to HIV infection. On the other hand, elevated expression levels of HLA DR on CD4+T cells has been associated with progression to disease in HIV-1 infected subjects (Mahalingam M, 1995, Kammerer R, 1996, Kestens L, 1994, Peakman M, 1995). Furthermore, Saifuddin et al, have shown that elevated expression of HLA DR on CD4+T cells can increase HIV-1 transcription by increasing the activity of AP-1, an inducible transcription factor important in T cell activation and HIV-1 expression (Saifuddin M, 2000). Therefore, the finding that stimulation of CD4+T cells from individuals with CD4 868T allele leading to a significant increase in HLA DR expression could provide a mechanism for their rapid disease progression upon HIV-1 infection. This rapid disease progression could be due to increased HIV-1 viral replication and/or cell death of activated cells. Since AICD has been reported as one of the mechanism that can lead to immunodeficiency, we sought to investigate apoptosis as a possible mechanism for rapid CD4+T cell loss in HIV infected people with CD4 868T allele.
6.9 Apoptosis is a mechanism for CD4+T decline in individuals with CD4 868T allele.

The binding of gp120 to CD4 molecules on T cells can induce signals that lead to cell death by apoptosis. Apoptosis or programmed cell death is a form of cell death that is characterized by cell shrinkage, loss of membrane integrity, chromatin condensation and nuclear fragmentation (Kerr JF, 1972). In the context of HIV-1 infection, apoptosis is considered to be one of the mechanisms by which HIV-1 eliminates CD4+T cells. Indeed, spontaneous apoptosis has been observed in PBMCs from HIV-1 infected persons and not in PBMCs from HIV-1 uninfected subjects, suggesting that it is one of the mechanisms for CD4 T cell loss in such subjects (Groux H, 1992, Meyard L, 1992). In particular, the gp120/CD4 interaction has been reported by numerous studies to enhance the rate of apoptosis in activated CD4+ T cells (Cicala C, 2000, Finkel, TH, 1995; Ullrich CK, 2000). Based on this background, we hypothesized that gp120 induced apoptosis is a mechanism of rapid CD4+T cell decline in individuals with CD4 868T allele. In order to assess the role apoptosis plays in the cell death of A2.01/868T cells, A2.01, A2.01/868T and A2.01/868C cells were cross-linked with anti-CD4 and anti-CD3 monoclonal antibodies as described in the methods section. Twenty four hours after cell stimulation, both un-stimulated and stimulated cells were stained with annexin V. The annexin V assay is based on the observation that soon after initiating apoptosis, most cell types translocate the membrane phospholipid phosphatidylserine (PS) from the inner face of the plasma membrane to the cell surface. Once on the cell surface, PS can easily bind to Annexin V, a protein that has a strong natural affinity for PS (Martin, SJ, 1995). The results from these assays showed a higher percentage of A2.01/868T cells undergoing cell death due to apoptosis compared to A2.01/868C cells (Figure 24). These results suggested that gp120/CD4 specific signals induced more or stronger death signals in the A2.01/868T cells than in the A2.01/868C cells. These results are in
agreement with other previous findings that had suggested apoptosis as a major contributor in the loss of CD4+T cells in HIV-1 infected persons (Ameisen JC, 1998, Terai C, 1991, Ameisen JC, 1991). Although several HIV-1 viral proteins or gene products can cause apoptosis in HIV-1 infected persons, the focus of this thesis is on gp120-induced apoptosis. We have demonstrated that 868T cells are more susceptible to apoptosis induced by AICD. Moreover, gp120 of HIV can induce apoptosis CD4+T cells independent of AICD. The question of whether gp120-induce apoptosis is the major contributor of rapid CD4+T cell loss in HIV-1 infected persons with CD4 868T allele is still unknown. However, given the fact that gp120 is found in different forms in HIV-1 infected people, it could bind to both HIV-1 infected and HIV-1 uninfected CD4+T cells. Indeed, gp120 has been shown to kill both HIV-1 infected as well as HIV-1 uninfected CD4+T cell in HIV-1 infected persons. For HIV-1 infected cells, the env expressed on HIV-1 infected cells can interact with HIV-1 uninfected cell and appropriate chemokine receptor causing syncytia and a subsequent cell death (Sodrosky JG, 1986, Ferri, KF, 2000). The syncytia-induced cell death was not investigated in this study. In addition to killing infected CD4+T cells, the env protein found either as free or virus-associated can bind to HIV-1 uninfected and cause cell death through activation induced apoptosis. Taken together, by targeting both HIV-1 infected and uninfected CD4+T cells, env-induced apoptosis could contribute to accelerated cell death in HIV-1 infected persons with CD4 868T allele and resulting in rapid disease progression.

The mechanisms by which gp120 cause the death of CD4+T cells via apoptosis have been described. Binding of gp120 to either CD4 or CXCR4 has been previously shown to induce apoptosis through several apoptotic signaling pathways (Figure 25). Gp120/CD4 interaction induces apoptosis by signaling through the CD95/CD95L-dependent death receptor pathway or,
Alternatively, through the Bax-dependent mitochondrial pathway to apoptosis (Hashimoto F, 1997, Somma F, 2000). Since the Bax-dependent signaling pathway to apoptosis is dependent on lck activity, it is possible that the mechanism for apoptotic cell death in A2.01/868T cells could be due to signals transduced through this pathway. We have shown from immune activation studies in this thesis that stimulation of A2.01/868T cells lead to a higher phosphorylation of lck in these cells compared to CD4 868C cells. While we were able to show more phosphorylation on lck negative regulator, tyr505, this phosphorylation could be transient, allowing more phosphorylation on the positive lck regulator, tyr394.

In conclusion, our studies suggest that apoptosis is one of the mechanisms that is playing a role in rapid CD4+T cell decline in HIV-1 infected persons with CD4 868T allele.

7.0 Conclusion and summary

Our study is the first study to evaluate the impact of the CD4 868 polymorphism on the functions of CD4. This study has revealed important novel mechanisms for both susceptibility to HIV-1 infection and the mechanisms for rapid disease progression in persons with CD4 868T allele. We know from this study that the increased susceptibility of individuals with CD4 868T allele to HIV infection is due their cells being more susceptible to HIV infection. We also know from this study that CD4-Trp240(868T) has reduced binding affinity to gp120 compared wild-type CD4. We also know that although gp120 binds to CD4-Trp240(868T) with lower affinity, the signals transduced through the interaction can turn on the CD4+T cells to become activated and either die due to apoptosis or become targets for HIV-1 infection. Since activation-induced cell death plays an important role in the pathogenesis of HIV-1, we strongly believe that this is one of the
major mechanisms for rapid CD4+T cell loss in HIV-1 infected persons with CD4 868T allele. In addition to providing a detailed mechanism for risks to HIV-1 infection and disease progression, there are two important lessons to learn from this study. First, given the high prevalence of CD4 868T and high prevalence of HIV-1 in people of Africa decent, we can speculate that CD4 868T is one of the host genetic factors that may have contributed to the dramatic spread of the virus in sub-Saharan Africa. Secondly, this study has provided new information that can be used for the development of newer and more effective antiretroviral drugs. The development of effective antiretroviral therapy that inhibit, HIV-1 from binding to chemokine receptors will depend on the deeper understanding of the events before and during gp120 binding to these HIV-1 co-receptors. As we have seen in this study that gp120 of HIV-1 binds to CD4-Trp240(868T) with lower affinity this may have an impact on the efficacy of the current and future CCR5/CXCR4 inhibitors, especially HIV-1 fusion inhibitors. Given that the majority of persons with the CD4 polymorphism are found in the regions of the globe with the highest HIV-1 prevalence, information from this thesis may prove useful hints in designing better chemokine receptor inhibitors.

Lastly, we made intriguing observations in this study that suggest apoptosis and activation-induced cell death are two major possible mechanisms for CD4+T cell loss in HIV-1 infected persons with the CD4 868T allele. Because apoptosis is a known mechanism of CD4+T cell decline in HIV-1 infected persons, understanding apoptotic mechanisms, especially those induced by viral proteins, may be important for the development of anti-HIV treatment.
8.0 Future Work

Although we made a number of findings on the molecular mechanisms of the CD4 polymorphism on susceptibility to HIV infection and disease progression, there are some gaps in knowledge that still need to be investigated. The following investigations will help in further understanding the molecular basis of CD4 polymorphism on either susceptibility to HIV-1 infection or its impact on disease progression; 1) investigate the impact of gp120-CD4-Trp240(868T) binding on the binding affinity of gp120 to either CCR5 and CXCR4. 2): Investigate if of CD4 polymorphism has greater impact individuals with homozygous 868TT with respect to HIV infection, activation status and the rate of apoptotic cell death. 3) The role of apoptosis in PBMCs of HIV-1 infected persons who have CD4 868T allele. 4) To investigate the impact of gp120-CD4-Trp240(868T) has on the either CCR5 or CXCR4 signaling on CD4 cells.
9.0 Study models.

Figure 25: The proposed model for CD4 868T increased susceptibility to HIV infection. The proposed study model shown above, suggests that exposure of CD4 868T cells to either gp120 or HIV induces signals that result in a higher activation level than in CD4 868C cells. Since HIV infects activated cells more efficiently than resting cells, having more activated cells provide more target cells for HIV infection, thus more virus production. Similarly, the enhanced activation of CD4 868T cells due to the interaction of gp120 to CD4-Trp240(868T) can result in more cell death either by direct apoptosis or activation-induced cell death. Therefore according to this model, individuals with CD4 868T allele are more susceptible to HIV infection due to their CD4+T cells ease of activation. Upon infection, these individuals are likely to loss CD4+T cells by apoptosis as well as by activation induced cell death.
Figure 26: The proposed model for CD4 868T rapid progression to HIV/AIDS disease. During HIV-1 replication both CD4 and gp160 are synthesized in the Endoplasmic Reticulum (ER). Since CD4-Trp240(868T) binds to gp160 with low binding affinity, it is expected that even if they form a complex, more gp160 will be released in Golgi apparatus to undergo cleavage into gp120 and gp41. On the other hand since wildtype CD4 binds to gp160 with higher affinity most gp160 will be trapped in ER in the form CD4-gp160 complex. At the cell membrane, since there are more gp120-gp41 in CD4 868T cells more virions will be formed and released (higher viral load). On the hand CD4 868C cells have on the surface CD4 that binds to the few virions formed and tither them (low viral load). Higher viral load is associated with rapid progression to AIDS in HIV infected patients.
10. Reference:


Collins TL, Burakoff SJ. Tyrosine kinase activity of CD4-associated p56lck may not be required for CD4-dependent T-cell activation. Proc Natl Acad Sci U S A. 1993 Dec 15;90(24):11885-9


### 11.0 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABS</td>
<td>Antibody binding sites</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-Dependent Cellular Cytotoxicity</td>
</tr>
<tr>
<td>AE</td>
<td>Elution Buffer (QIAamp DNA Mini Kit)</td>
</tr>
<tr>
<td>AICD</td>
<td>Activation-induced cell death</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>AL</td>
<td>Lysis Buffer (QIAamp DNA Mini Kit)</td>
</tr>
<tr>
<td>Am</td>
<td>Ammonium Persulfate</td>
</tr>
<tr>
<td>ARVs</td>
<td>Anti-Retro Viral</td>
</tr>
<tr>
<td>AW1</td>
<td>Wash Buffer (QIAamp DNA Mini Kit)</td>
</tr>
<tr>
<td>BD</td>
<td>Beckton Dickinson</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CCR2</td>
<td>Chemokine Receptor 2</td>
</tr>
<tr>
<td>CCR5</td>
<td>Chemokine Receptor 5</td>
</tr>
<tr>
<td>CCR5-Δ32</td>
<td>Chemokine Receptor 5 delta 35</td>
</tr>
<tr>
<td>CD3</td>
<td>Cluster Differentiation 3</td>
</tr>
<tr>
<td>CD4</td>
<td>Cluster Differentiation 4</td>
</tr>
<tr>
<td>CD4-Trp240</td>
<td>Cluster Differentiation 4, Tryptophan 240</td>
</tr>
<tr>
<td>CD8</td>
<td>Cluster Differentiation 8</td>
</tr>
<tr>
<td>CDC</td>
<td>Centres for Disease Control</td>
</tr>
<tr>
<td>CTLs</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CXCL12</td>
<td>Chemokine (C-X-C motif) ligand 12</td>
</tr>
<tr>
<td>CXCR4</td>
<td>CXC Chemokine Receptor</td>
</tr>
<tr>
<td>DAR C</td>
<td>Duffy antigen/chemokine receptor</td>
</tr>
<tr>
<td>DEA</td>
<td>diethanolamine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescent</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine Tetracetic Acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbant assay</td>
</tr>
<tr>
<td>Env</td>
<td>Envelope</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Gag</td>
<td>Group Specific Antigen</td>
</tr>
<tr>
<td>GAM</td>
<td>Goat Anti-Mouse</td>
</tr>
<tr>
<td>gp120</td>
<td>Glycoprotein 120</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>HCL</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigens</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>HTLV-III</td>
<td>Human T-Cell Leukemia virus</td>
</tr>
<tr>
<td>IDU</td>
<td>Injection Drug Users</td>
</tr>
<tr>
<td>IL-16</td>
<td>Interleukine 16</td>
</tr>
</tbody>
</table>
IP: Immuno precipitation
IU: Infectious Units
KC57: An antibody that identifies the p55, p39, p33 and p24 proteins of HIV-1.
Kd: Kilodalton.
LAV: Lymphadenopathy-Associated Virus
Lck: Lymphocyte-Specific protein tyrosine Kinase.
LN: lymph nodes
LTNP: Long-Term Non-Progressors
LTR: long terminal repeat
Luc: Luceferase
MA: Matrix.
MAC: Mycobacterium avium complex
ME: 2-Mercaptoethanol
MgCl2: Magnesium Chloride.
MMWR: Morbidity and Mortality weekly report.
MOI: multiplicity of infection
NaCl: Sodium Chloride
NaOH: Sodium hydroxide.
NIAID: National Institute of Allergy and Infectious Diseases.
nM: Nanomole.
nsSNPs: non-synonymous Single Nucleotide Polymorphism.
OKT4: Ortho Kung T4.
OPV: Oral Polio Vaccine
p24: Protein 24.
PBMCs: Peripheral Blood Mononuclear Cells
PBS: Phosphate Buffered Saline
PCP: Pneumocystis pneumonia.
PCR: Polymerase chain reaction
PE: Phycoerythrin
PFA: Paraformaldehyde
PHA: Phytohaemagglutinin
PML: Progressive multifocal leukoencephalopathy
RANTES: Regulated upon activation, normal T-cell expressed, and presumably secreted.
RNA: Ribonucleic acid.
RPMI 1640: Rosewell Park Memorial Institute 1640.
SAAP: Streptavidin-Alkaline-Phosphatase.
SDF1: Stromal cell-mediated factor 1.
SDS-PAGE: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis.
SIM.4: A hybridoma for anti-CD4(Leu-3a).
SIV: Simian Immunodeficiency Viruses
SLE: Systemic Lupus Erythematosus.
SNP: Single Nucleotide polymorphism.
Src: a family of proto-oncogenic tyrosine kinases
STIs: Sexually transmitted infection
Taq: Thermus aquaticus
TAT: transcriptional trans-activator protein.
TCA: Trichloroacetic Acid.
TCID$_{50}$: Tissue culture infectious dose 50.
TCR: T cell antigen receptor
TEMED: Tetramethylethylenediamine.
TRIS: tris (hydroxymethyl) aminomethane.
Vif: Virus Infectivity Factor
Vpr: Virul protein r
Vpu: Virus protein u
VSV.G: vesicular Stomatitis Virus glycoprotein
WHO: World Health Organization.