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Role of Cellular Immune Functions through the Course of HIV-1 Natural Infection in Ugandans

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Obu niburwaireki oburikwita omushaija, bukataho omukazi n'abaana enju nakagisiba? Pe!

Translation: *What kind of monster disease is this that kills a man, his wife and all their children, thus closing the entire homestead?*

Yudesi Ndimbirwe, 1989
From: *Genocide by Denial* by Peter Mugenyi, 2008

…if you do not know your enemies nor yourself, you will be imperiled in every single battle.

Thus, what is of supreme importance in war is to attack the enemy's strategy.

Sun Tzu, The Art of War 6th century B.C.

ABSTRACT

HIV-1 infection remains a major crisis in sub-Saharan Africa and more information about disease pathogenesis and immune correlates of protection are needed. Uganda, with a population of approximately 33 million people, has a national HIV-1 prevalence over 6% with subtype A and subtype D predominating. We aimed to characterize immune cell functions in Ugandans with untreated chronic HIV-1 infection, and identify aspects of the immune response that are associated with control of viremia and disease progression. As this work was based on stored specimens from cohorts in the rural districts of Kayunga and Rakai, we first detailed the importance of rigorous protocols for quality PBMC cryopreservation in the resource limited setting in **Paper I**. Importantly, cryopreservation did not compromise relative frequencies or function of PBMCs, and long-term storage of samples for greater than 3 years did not impact yield or viability. We developed a program to monitor PBMC processing to ensure suitability for the studies of adaptive and innate immunity included in this thesis. In **Paper II**, we found redistribution of NK cell subsets, with increase in $CD56^{neg}$ NK cells and reduction of $CD56^{dim}$ NK cells, in HIV-1 infected Ugandans. Moreover, we observed decreased NK cell expression of KIR2DL1, NKG2A, CD161 and NKp30 in these patients. Interestingly, severe loss of CD4 T cells was associated with elevated levels of KIR expression and degranulation in $CD56^{bright}$ NK cells, suggesting that cytotoxic function develops in this subset in progressive HIV disease. In **Paper IV**, we continued to build on these findings and discovered a preferential expansion of KIR3DL1+ NK cells that was directly proportional to HIV-1 viral load in donors that possessed the HLA-B Bw4-80I motif. Other inhibitory KIRs were reduced or remained constant in the presence of their HLA ligands. Overall, NK cells in HIV-1 infected Ugandans displayed an elevated activity despite an altered functional and phenotypic profile in chronic disease. Additionally, NK cells in these patients were more polyfunctional with regard to CD107a, IFN-γ, and MIP-1β expression as compared to uninfected controls. The KIR3DL1+ NK cells in Bw4+ individuals were particularly responsive, producing increased IFN-γ and MIP-1β. In **Paper III**, we examined T cell activation in HIV-1 infected Ugandans, in an effort to better define the phenotypic aspects unique to progressive infection and understand the mechanisms behind disease progression. We found that activated CD4 T cells displayed a deregulated effector memory (T_{DEM}) phenotype and levels of such cells were directly proportional to HIV-1 viral load. Individuals with elevated frequencies of CD4 T_{DEM} cells progressed faster to AIDS. These CD4 T_{DEM} cells correlated with markers of microbial translocation and innate immune activation such as sCD14 and IL-6. *In vitro* assays revealed that CD4 T_{DEM} cells displayed a diverse TCR Vβ repertoire, and could be driven by a diverse array of pathogens including HIV-1 itself. Taken together, the CD4 T_{DEM} cell data supports a model where innate immune activation and chronic antigen stimulation are involved in pathological T cell activation and HIV-1 disease progression. In summary, these Ugandan cohort studies have provided insight into the balance between healthy immune responses and pathological immune activation that characterizes HIV-1 infection. More targeted studies are needed in order to develop therapeutic and preventative strategies that may alleviate the burden of HIV-1.

LIST OF PUBLICATIONS

This is based on the following papers, which are referred to in the text by their Roman numerals.

I. Quality Monitoring of HIV-1 Infected and Uninfected Peripheral Blood Mononuclear Cell Samples in a Resource Limited Setting.
Robert E. Olemukan, Leigh Anne Eller, Benson J. Ouma, Ben Etonu, Simon Erima, Prossy Naluyima, Denis Kyabaggu, Josephine H. Cox, Johan K. Sandberg, Fred Wabwire-Mangen, Nelson L. Michael, Merlin L. Robb, Mark S. de Souza, **Michael A. Eller.**
Clinical and Vaccine Immunology, June 2010, Vol. 17, No. 6, p. 910 – 918

II. Elevated NK Cell Activity despite Altered Functional and Phenotypic Profile in Ugandans with HIV-1 Clade A or Clade D Infection.
Michael A. Eller, Leigh Anne Eller, Benson J Ouma, Doris Thelian, Veronica D Gonzalez, David Guwatudde, Francine E McCutchan, Mary A Marovich, Nelson L Michael, Mark S de Souza, Fred Wabwire-Mangen, Merlin L Robb, Jeffrey R Currier, Johan K Sandberg.
Journal of Acquired Immunodeficiency Syndrome, August 2009, Vol. 51, No.4, p. 380 – 389

III. Innate and Adaptive Immune Responses Both Contribute to Pathological CD4 T Cell Activation Predictive of Disease Progression in HIV-1 Infected Ugandans.
Michael A. Eller, Kim G Blom, Veronica D Gonzalez, Leigh A Eller, Prossy Naluyima, Oliver Laeyendecker, Thomas C Quinn, Noah Kiwanuka, David Serwadda, Nelson K Sewankambo, Boonrat Tasseneetrithep, Maria J Wawer, Ronald H Gray, Mary A Marovich, Nelson L Michael, Mark S de Souza, Fred Wabwire-Mangen, Merlin L Robb, Jeffrey R Currier, Johan K Sandberg.
PLoS One, in press.

IV. Human Immunodeficiency Virus Type 1 Infection is Associated with Increased NK Cell Polyfunctionality and Higher Levels of KIR3DL1+ NK Cells in Ugandans Carrying the HLA-B Bw4 Motif.
Michael A. Eller, Rebecca N. Koehler, Gustavo H. Kijak, Leigh Anne Eller, David Guwatudde, Mary A Marovich, Nelson L Michael, Mark S de Souza, Fred Wabwire-Mangen, Merlin L Robb, Jeffrey R Currier, Johan K Sandberg.
Journal of Virology, in press.

TABLE OF CONTENTS

FOREWORD

Throughout my studies and professional career, the HIV/AIDS epidemic has driven and stimulated scientific advancement to better understand the intricacies between host and pathogens. A safe and effective HIV vaccine is desperately needed and the search continues. Over the past 14 years I have participated in HIV trials and cohort development via technical support and immunogenicity evaluation of several products and natural history protocols. As an employee of the US Military HIV Research Program (MHRP), I have experienced the challenges of conducting human studies and understand the paramount need to discover correlates of protection. Our understanding of immune response manipulation through HIV vaccination continues to expand with every clinical trial that is conducted. In 2003, I moved to Kampala, Uganda, where I was responsible for development of PBMC processing and immunological studies for the Makerere University Walter Reed Project (MUWRP), part of the MHRP network. Living in Uganda, I witnessed a different perspective on the impact of HIV on the population and the foundation of society. Here, I was able to study T-cell immune responses to DNA and adenovirus vaccine platforms developed by the Vaccine Research Center, National Institutes of Health. In addition to human clinical trials, natural cohort studies have provided a crucial understanding of how HIV-1 interacts with our immune system. It is here that I began a new chapter in my life.

In 2007, I entered into a PhD program through the Center for Infectious Medicine, at the Karolinska Institutet in Stockholm, Sweden, and through a tri-continent coalition the studies mentioned in this thesis were outlined and executed. This collaborative initiative began by describing the importance of PBMC processing and means to ensure quality in order to support downstream immunological assessment. More recently I have investigated how the innate immune population of NK cells respond during chronic HIV-1 infection and continue to explore activating and inhibitory receptors with intense interest in KIRs and their HLA ligands. Harnessing this innate effector population may help vaccine design and development. Through our cohorts, a number of studies were carried out on T cells as well. This thesis will discuss the aberrant T cell activation that has been intimately associated with HIV-1 disease progression. It is my hope that the studies below will not only provide additional insight into the HIV-1 epidemic in Uganda with regard to cellular immunity, but also generate new ideas and hypothesis to test in future studies.

I am now living back in the US and working at the MHRP in Rockville, Maryland. Within the Department of Vaccine Research and Development there is an excitement and a sense of urgency to capitalize on the recent Thai phase III RV144 trial and identify possible correlates of protection. The work I have completed in Africa, Sweden and the US has allowed me to broaden my horizons, establish collaborations and help develop my career as an independent research scientist in the search for a correlate of protection for HIV.

Michael A. Eller Stockholm, April 2, 2011

ABBREVIATIONS

Ad5	adenovirus serotype 5
ADCC	antibody dependent cellular cytotoxicity
AIDS	acquired immune deficiency syndrome
ART	anti-retroviral therapy
AZT	azidothymidine
CCR7	CC chemokine receptor 7
CD	cluster of differentiation or cluster of designation
CDC	Centers for Disease Control
CFSE	carboxyfluorescein diacetate succinimidyl ester
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
CTLA	cytotoxic T lymphocyte antigen
EBV	Epstein-Barr virus
DAMP	danger associated molecular patterns
DC	dendritic cell
FACS	fluorescence-activated cell sorting
FasL	Fas ligand
FOXP3	forkhead box protein 3
GALT	gut associated lymphoid tissue
HAART	highly active anti-retroviral therapy
HEV	high endothelial venules
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPV	human papillomavirus
ICOS	inducible T cell costimulator
IFN	interferon
IL	interleukin
ITAM	immunoreceptor tyrosine-based activating motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
ITSM	immunoreceptor tyrosine-based switch motif
KIR	killer cell immunoglobulin-like receptors
LAMP	lysosomal-associated membrane protein
LFA	lymphocyte function associated antigen
LPS	lipopolysaccharide
MALT	mucosal associated lymphoid tissue
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
NCAM	neural cell adhesion molecule
NCR	natural cytotoxicity receptors
NK	natural killer
NOD	immunoreceptor tyrosine-based inhibitory motif
PAMP	pathogen associated molecular patterns
PBMC	peripheral blood mononuclear cells
PD-1	programmed death receptor-1
pDC	plasmacytoid dendritic cell

PDL	programmed death ligand
PFC	polychromatic flow cytometry
PMT	photo-multiplier tube
PRR	pattern recognition receptor
RAG	recombination-activating gene
SEB	staphylococcal enterotoxin B
SIV	simian immunodeficiency virus
SLAM	signaling lymphocytic activation molecule
TLR	Toll-like receptors
TNF	tumor necrosis factor
TRAIL	(TNF)-related apoptosis-inducing ligand
T_{FH}	follicular helper T cells
T_{regs}	regulatory T cells
WHO	World Health Organization

1. HIV-1 AND AIDS

1.1 Overview

In 2008, two French scientists were selected by the Karolinska Institutets Nobel Assembly to be awarded half of the *Nobel Prize in Physiology or Medicine* for their identification of the virus that was later shown to cause acquired immune deficiency syndrome (AIDS). In this seminal work by Francoise Barre-Sinoussi and Luc Montagnier, a new retrovirus was isolated from a patient's lymph node that had similar features to a family of viruses known as human T-cell leukemia viruses[1]. A subsequent study in the US, lead by the efforts of Robert Gallo, found that in a significant number of patients with symptoms that precede AIDS or in patients with AIDS defining illness, a virus he and colleagues named human T-lymphotropic retrovirus III (HTLV-III) was frequently isolated from peripheral blood lymphocytes supplemented with T cell growth factor[2]. From these pivotal studies and subsequent work, it became clear that the virus we now know as the human immunodeficiency virus (HIV), was the cause of AIDS. This disease was first characterized in homosexual men and drug users between 1979 and 1981 who acquired *Pneumocystis carinii* Pneumonia and was associated with an immune dysfuntion[3]. Over the next three decades, scientists made significant progress in immunology, virology, and through technical advancement our understanding of HIV-1 has grown. Despite the enormous efforts around the globe, HIV and AIDS has become one of the worst epidemics of all time. Currently, there are over 33 million people living with HIV-1 while an estimated 1.8 million people die each year from AIDS related illness. Almost two-thirds of this pandemic resides in Sub-Saharan Africa, a setting limited in the resources necessary to combat the disease[4].

1.2 HIV-1 virology

HIV-1 is a single stranded, positive-sense enveloped RNA virus from the family Retroviridae, subfamily Orthoretroviridae. HIV-1 virions are spherical and 80-110 nm in diameter encased by a lipid-containing envelope with glycopeptide spikes 8 nm in length that surround an icosohedral shaped capsid. HIV-1's RNA genome is relatively small, approximately 9 kilobases, and encodes for 14 proteins including 3 structural proteins, 2 envelope proteins, 6 accessory proteins, and 3 enzymes all of which facilitate entry, reverse transcription, integration and replication within the host's cells[5]. Figure 1 summarizes the complete replication cycle of HIV-1. The first step in the replication cycle of HIV-1 is binding and entry into the target cell through the cluster of differentiation (CD)4 receptor, first characterized on a subset of T cells[6,7]. Other cell types such as dendritic cells (DC) and macrophages have been shown to be infected and could play a crucial role in viral dissemination, particularly in the case of DC[8-11]. HIV-1 binds the CD4 receptor, a surface glycoprotein, resulting in a conformational change that allows co-receptor binding, membrane fusion and injection of the viral capsid into the cytosol[12]. Once inside the cell, HIV-1's RNA genome is reverse transcribed into double stranded DNA by the viral enzyme reverse transcriptase (RT) and is transported inside the nucleus where the integrase enzyme incorporates the viral DNA into the host DNA thereby establishing infection[13]. The proviral DNA can remain latent or upon activation, polymerase II can initiate the transcription and creation of mRNA transcripts. The transcripts are targeted out of the nucleus to various compartments in

the cytosol where they are translated and transported to the cell surface for assembly[5]. Immature virions bud from the surface of the cell enveloped in host cell lipid bilayer, other surface receptors, and viral glycoprotein spikes, then, finally mature into infectious virions[13].

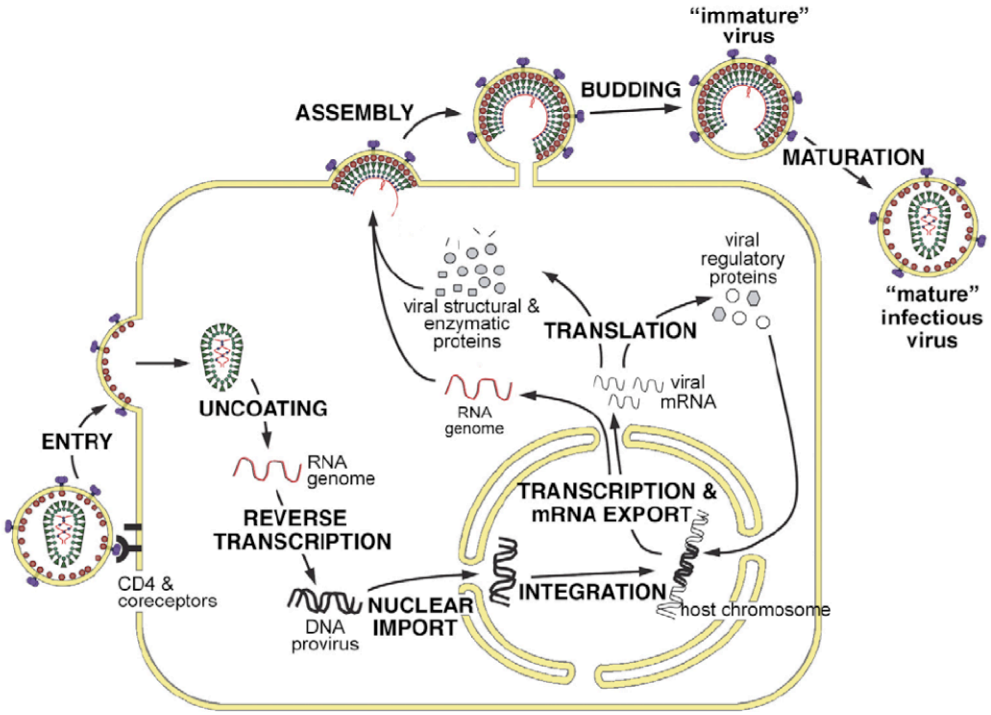

Figure 1. HIV-1 Replication Cycle (adapted from Ganser-Pornillos, B.K. *et al.*, 2008)[13].

The origin of HIV is thought to have occurred from human contacts with non-human primates in Africa. HIV-1 and HIV-2 represent two separate cross-species transmissions from the common chimpanzee (*Pan troglodytes troglodytes*) and sooty mangabey (*Cercocebus atys*) respectively, based on HIV phylogenetic lineage characterization[14]. HIV-2 is less prevalent compared to HIV-1, is less efficiently transmitted and results in a slower disease progression[15]. HIV-1 is the virus responsible for the global pandemic and has a high rate of replication, transcriptional error and recombination, resulting in great genetic diversity throughout the world. Phylogenetic analysis identifies three major groups of HIV-1 labeled as M, N, and O, where group M represents the main group with the greatest number of variations that are clustered together into distinct lineages called subtypes or clades[16]. There are 9 known subtypes of HIV-1 group M virus, A-D, F-H, J-K and a growing number of recombinant forms are developing around the world that further contribute to the global diversity of HIV-1[17]. HIV-1 subtype B is predominant in the US and Europe, subtype AE is found in southeast Asia, subtype A, C and D are most common in East Africa while infections in South Africa are predominantly subtype C. Globally, subtype C is the most prevalent, accounting for about 50% of infections[18]. Another way to classify virus is based upon coreceptor utilization. The primary coreceptors, CXCR4 and CCR5, are able to classify virus as macrophage infecting (M-tropic), T cell infecting (T tropic) or both (dual tropic)

(reviewed in[19]). The extreme diversity of HIV-1 contributes to the difficulty in development of measures to prevent acquisition of this virus.

1.3 HIV-1 clinical features and disease progression

HIV-1 is predominantly transmitted sexually through genital or rectal mucosa, although direct infection through intravenous drug use is also a common mode of transmission among certain populations. HIV-1 infects cells at the mucosal barrier, targeting CD4+ T cell and dendritic cell rich areas often found in the cervico-vaginal region in women and in the inner foreskin and penile urethra of men[20]. Once infection occurs at the mucosal sites, dendritic cells, among others, are responsible for transporting virus away from the site of transmission to the draining lymph node where additional targets are found for subsequent rounds of viral amplification. This period is known as the eclipse phase of infection, which is typically less than 10 days before viral RNA becomes detectable in the plasma[21]. New advances in diagnostic technology allow for the staging of acute HIV-1 infection from Fiebig I-V during the first 100 days of infection and are differentiated by the acquisition of detectable viral RNA, antigen reactivity, and followed by detectable antibody responses[22]. Peak HIV-1 viremia occurs approximately 20 to 30 days after infection and coincides with a wide distribution of virus through out the body including the gut associated lymphoid tissue (GALT) where an abundance of target CD4+ T cells reside[21]. Widespread CD4+ T cell depletion is a hallmark of HIV infection and results in breakdown of physical and chemical barriers at certain sites, such as the GALT, leaving the host immune compromised during primary infection. Common signs and symptoms of HIV-1 primary infection include fever, myalgia, lymphadenopathy, headache, nausea, diarrhea, vomiting and rash[23]. In most cases, these effects subside concurrent to host viral control and the establishment of set point viremia follows, leading to early chronic infection and temporary stabilization of the immune compartment.

Chronic infection is associated with an asymptomatic period that varies in length based on host and viral determinants, environmental factors and behavior traits that may all contribute to disease progression. A typical course of chronic infection may last up to 12 years and is associated with a delicate balance of viral replication, CD4+ T cell depletion and regeneration that the immune system is unable to sustain, resulting in the development of AIDS[24]. The World Health Organization (WHO) classifies disease progression based on presentation of specific signs or symptoms[25]. Clinical stage I is characterized as asymptomatic or persistent generalized lympadenopathy with various degrees of weight loss and opportunistic infections[25]. The most severe phase, clinical stage IV, may result in *Pneumocystis carinii* pneumonia, Toxoplasmosis of the brain, Cryptosporidiosis with diarrhea for more than a month, Kaposi's sarcoma and other serious conditions[25]. A similar staging system, from the Centers for Disease Control and Prevention (CDC), exists based on CD4+ T cell counts with >500 cells/μl of whole blood corresponding to less severe disease and CD4+ T cell counts with <200 cells/μl qualifying as AIDS[26]. Left untreated, AIDS results in death due to opportunistic infections that the host is unable to combat.

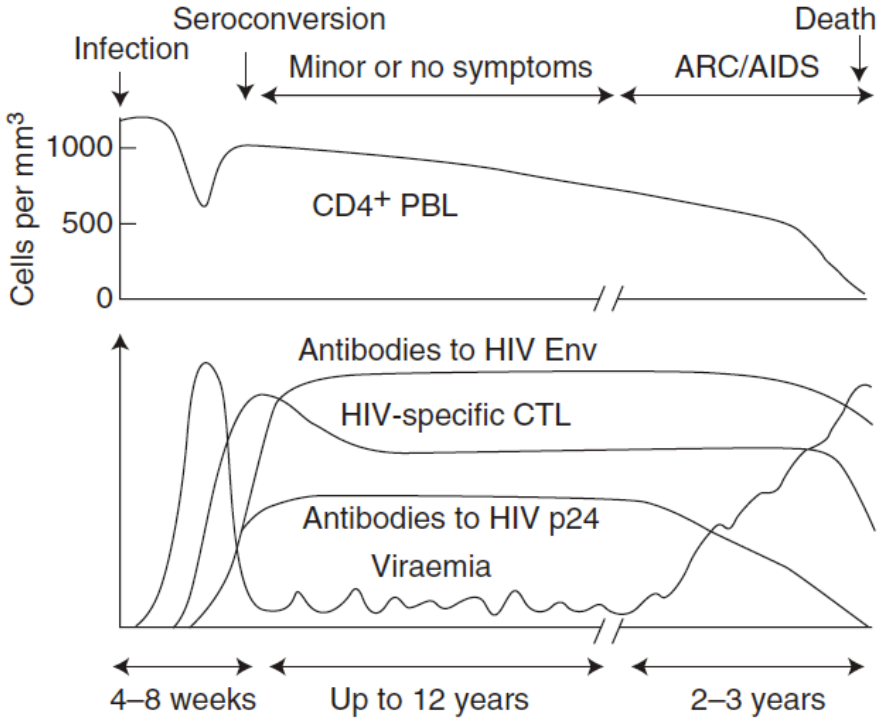

Figure 2. HIV-1 disease progression (adapted from Weiss, R.A., 2008)[24].

1.4 HIV-1 treatment and prevention

After the identification of the virus that causes AIDS, extraordinary scientific efforts were made to develop diagnostic techniques to detect infection and understand the replication cycle of HIV-1 in order to identify potential therapeutic targets that mitigate the disease. Early clinical trials tested azidothymidine (AZT), a nucleoside analog reverse transcriptase inhibitor that was effective in reducing HIV-1 viral load, but despite the initial success, patients quickly developed drug resistant strains to this monotherapy[15]. Additional pharmaceuticals such as protease inhibitors, alternative reverse transcriptase inhibitors, integrase inhibitors, and cell entry inhibitors were designed and developed to interrupt different stages of the HIV replication cycle. These drugs, when used in combinations of three or more, were termed highly active anti-retroviral therapy (HAART) and proved to be a much more effective treatment regimen[24]. HAART became widely available to HIV-1 infected patients in countries that could afford the high cost of these drugs and more recently have been made available in resource-limited settings. HAART reduces HIV-1 viral load upon treatment initiation and gradual increases in CD4+ T cells are observed throughout successful therapy. The use of HAART has reduced AIDS related deaths, but there is continued debate over how early to initiate treatment as some studies suggest that earlier treatment intervention leads to better outcomes[27]. However, the benefits of initiating HAART during acute HIV-1 infection are unclear[28,29]. HAART is not without limitations. Some individuals continue to experience CD4+ T cell decline despite successful viral suppression potentially caused by the irreversible damage to the T cell compartment in early infection[30]. In addition, drug toxicities, side effects and cost are hurdles that need to be overcome. Despite the clear evidence that starting patients on HAART early is beneficial, this

remains a daunting task in settings where access to these life saving drugs remain inadequate. Alternative strategies to prevent the acquisition of HIV-1 are urgently needed, as we may not be able to treat our way out of this epidemic[31-33].

A number of measures have been explored in order to prevent the acquisition of HIV-1, with the ultimate goal of a safe and effective vaccine. Many challenges and hurdles have plagued these efforts, but several successes have also been realized over the past decade. One major prevention strategy was to educate and alter behaviors in order to reduce HIV-1 incidence. The ABC's were an example of teaching people to "A" - abstain from sex, "B" - be faithful to your partner, and "C" use condoms. It is hard to measure the success of such prevention strategies, but this message remains a central mantra, particularly in Africa where the rate of new infections exceeds the rate of patients initiating treatment[15]. Medical male circumcision is another approach to prevent HIV-1 infection and three randomized, controlled trials in Africa exhibited a reduction in transmission of 53% - 60% in men undergoing the surgical procedure[34-36]. However, the long-term population effect of male circumcision remains conjecture and rolling out widespread surgical interventions in Africa presents an enormous challenge[37].

The most desired prevention method for the majority of infectious diseases, vaccination, had proven not only unsuccessful in the HIV-1 field, but also unlikely, until a groundbreaking proof of concept trial in 2009. A community based randomized, double-blind, placebo-controlled efficacy trial of HIV-1 canarypox vector prime, boosted with recombinant glycoprotein 120 (gp120) subunit vaccine in Thailand exhibited a modest and transient efficacy in protecting trial participants[38]. While the results of the RV144 trial demonstrated that an HIV-1 vaccine is possible, the field is still years away from developing a product that is ready for mass distribution. In 2010, another randomized prevention trial provided a new weapon into our arsenal for HIV-1 prevention. Pre-exposure of men who have sex with men with a daily regimen of two antiretroviral drugs, emtricitabine and tenofovir disoproxil fumarate, showed a 44% reduction in HIV-1 acquisition[39]. While these results are promising, a number of concerns remain with long-term adherence, drug toxicities, and drug resistant acquisition[40]. Taken together, our HIV-1 prevention repertoire remains limited and underscores the need for a better understanding of the complex interaction between host and virus in order to reduce HIV-1 incidence through more targeted interventions.

1.5 HIV-1 in Uganda – The Pearl of Africa

While the first reports of HIV-1 and AIDS were focused on men who have sex with men and intravenous drug users in the US and Europe, a completely different story was unfolding in Africa. Uganda, an Eastern African country west of Kenya and nestled atop Lake Victoria is approximately 250,000 square km with a population of over 33 million[41]. In 1985, David Serwadda and colleagues published an article in Lancet characterizing "Slim Disease" in the southwestern district of Rakai, Uganda[42]. In this article, a new disease associated with promiscuous heterosexual patients presenting with abnormal and prolonged weight loss, diarrhea, oral candidiasis, and other opportunistic infections was associated with what was later named HIV-1. Unlike the previous reports in the west, this disease was not associated with homosexual behavior and did not have the same prevalence of Kaposi sarcoma, and therefore was suspected

as being of unique origin. More knowledge and awareness regarding the HIV-1 epidemic followed and a national hospital based surveillance system showed that this was a disease of men and women primarily aged 15-42 years with most showing symptoms of weight loss, fever, diarrhea, cough, and rash[43]. HIV-1 prevalence was shown to be approximately 28% in 1991 and confirmed a widespread epidemic[44]. Through education and modification to sexual behavior, Uganda was considered a major success story as the HIV-1 prevalence rates dramatically reduced to a reported 12% in 1997[45], however a number of investigators caution about over interpreting the reasons for the decline[46,47]. Uganda quickly developed infrastructure to study HIV-1 natural history cohorts in order to better understand the dynamics of HIV-1 infection in the community[48,49]. Uganda continues to make progress with regards to lowering the national prevalence of HIV-1 as more recent reports estimate 6.4% of the population are HIV-1 infected[50] but data exists that HIV-1 could be on the rise again[51]. The HIV-1 epidemic in Uganda has evolved and additional prevention strategies are needed to reduce the number of new infections while treatment scale-up continues.

Early on, Uganda embraced research on prevention strategies to combat HIV-1 and the community responded by participating in a number of pivotal studies that have helped shape the understanding of the disease. Uganda was the first African country to participate in a preventative HIV-1 vaccine trial. An HIV-1 canary pox vaccine was tested for safety in a group of 40 HIV-1 uninfected Ugandans, and was determined to be safe and mildly immunogenic[52]. This opened up testing of many preventative HIV-1 vaccine strategies such as DNA alone[53], in combination with modified vaccinia virus Ankara (MVA)[54], or in combination with recombinant adenovirus serotype 5 (Ad5)[55]. While these trials demonstrate increased capacity to conduct clinical research in Uganda, the collective results show little advancement towards an effective vaccine. Another prevention strategy, a medical male circumcision trial in 4,996 uncircumcised men in Rakai District, Uganda showed 55% efficacy[36]. The community in Rakai continues to investigate the potential benefits of circumcision on transmission studies of human papillomavirus (HPV) from men to uninfected women where female partners of circumcised males were at lower risk to contract HPV [56]. Another study failed to show any effect of medical male circumcision of HIV-1 infected men to their uninfected female partner[57]. While circumcision provides some protection, Uganda currently lacks the infrastructure to support widespread implementation in the general population. Other interventions have been explored in at risk populations such as children born to HIV-1 infected mothers. Uganda has participated in studies to prevent vertical transmission of HIV-1 using administration of the antiretroviral drugs AZT in combination with lamivudine[58] or comparing AZT to nevirapine[59]. While the results of these studies show reduction in mother to child transmission, the issue of transmitting drug resistant strains of HIV-1 remains a concern[60]. Uganda has participated in a plethora of other clinical studies and has demonstrated that the people of Uganda are willing to take part in the study of diseases relevant to the population.

Figure 3. Map of Uganda.

In addition to human clinical trials, Uganda has shown great prowess and progress in developing the infrastructure to conduct basic scientific research and diagnostic testing due to the HIV-1 epidemic. Several studies examined more efficient and reliable ways to diagnose HIV-1 infection using rapid platforms[61,62]. Furthermore, technology to characterize the molecular epidemiology has shed light on the viral diversity in Uganda, which may impact disease progression and can complicate development of preventative and therapeutic interventions[63-66]. There is also a growing ability to conduct sophisticated immunology studies in the context of both vaccine immunogenicity[52-55] and HIV-1 natural cohort studies [67-71]. Taken together, Uganda has made extraordinary advancement in the face of adversity by embracing the research and development surrounding infectious diseases, in particular HIV-1. Despite these efforts, more information is required about factors driving disease and the host responses associated with more favorable outcomes that could be replicated or harnessed through modern medicine. Understanding the human immune system and the cells that mediate infection control could provide crucial insight into these matters.

2. THE HUMAN IMMUNE SYSTEM

2.1 Overview

Since the beginning of life, organisms have competed for the precious resources on this planet to sustain their existence and evolution facilitated the development of a range of complex systems that favor one organism over another. In fact, Charles Darwin's theory of natural selection depicted a situation where individuals with favorable characteristics would breed and survive while those organisms with less favorable characteristics would struggle for existence[72]. This holds true for the human immune system. Put simply, immunology is the study of the body's defense against infection. The human immune system has evolved to incorporate a number of strategies to protect from "enemies both foreign and domestic" (US Armed Forces OATH OF ENLISTMENT), and like any army, has an arsenal at its disposal. The foreign invaders we encounter include bacteria, fungi, parasites, and viruses, which are constantly trying to break through our barricades to infect and compete for our resources while domestic issues arise such as autoimmunity, hypersensitivity, immune deficiencies and tumors. All human immune components can trace back to lower level organisms; in fact all living organisms have what some would argue is a level of immune response. For example, the amoeba, which predates eukaryotic cells by billions of years, may be the ancestor of modern phagocytosis, a major component of immunity employed by macrophages or other antigen presenting cells which engulf and digest pathogens[73]. Another example would be the toll-like receptors (TLRs), which are specialized receptors able to recognize certain pathogen associated molecular patterns (PAMPs) and were named for their resemblance of a protein coded for by the *toll* gene in the fruit fly (genus *Drosophila*). Recently, a TLR was reported in sponges (*Suberites domuncula*) that recognize bacterial lipopolysaccharide (LPS), which signifies that this innate immune receptor and mechanism has been around for approximately 800 million years[74]. The fact that many components of the human immune system have ancestral homologs in other organisms is not surprising, but puts in context the time frame and evolutionary impact that has selected for the complexity, diversity and ability to deal with a wide range of pathogens and disease.

Immunology is classically a dichotomous field, segregating components into polar groups such as innate versus adaptive, cellular versus humoral, self versus non-self, lymphoid versus myeloid, and so on. To this extent, we may oversimplify or misinterpret the gray area in between many of these groups. As we develop a better understanding of the function and phenotype of particular responses, we can form better models that connect the bi-polar nature of immunology. However, it is important to break things down to understand how each component works and then try to put it together to see how the pieces integrate. The human immune system has 4 components that are responsible for protecting the host from disease: recognition, effector functions, regulation and memory[75]. There exist many components to the immune system such as cells, proteins, chemicals, and even physical barriers. Important in immunology is the ability to recognize a foreign pathogen or define danger while being able to discriminate and protect "self" and this is accomplished through specialized receptors on the surface of cells of the immune system. The components of the immune system are grouped

based on specific or nonspecific recognition, which corresponds to adaptive and innate immunity respectively. Innate immunity is an immediate response of cells with a repertoire of specialized pattern recognition receptors (PRR) specially designed to recognize PAMPs and contain infection. The adaptive immune response results from initial exposure to a pathogen leading to the stimulation and priming of a naïve cell highly specific for that pathogen followed by expansion and development of immunologic memory. Upon re-exposure, the adaptive immune system can contain and eliminate the pathogen with greater efficiency. The interplay between infection and immune response is intricate and it is important to understand how the cells of both innate and adaptive arms work to better define how immune responses develop and what may be critical to provide protection from disease.

2.2 Innate immunity

Innate immunity consists of germline encoded, non "antigen-specific" cells that are able to respond to a diverse range of PAMPs in a quick and broad effort to clear infection or provide control until the adaptive immune response can support and clear the pathogen. A number of innate mechanisms have evolved to deal with the infectious burden that we constantly encounter. The innate immune response is comprised of a number of cells including DC, granulocytes, macrophages, monocytes, natural killer (NK) cells, and others that do not undergo clonal expansion or receptor rearrangement in order to recognize particular antigens. These cells can respond with a number of effector functions such as phagocytosis, production of cytokines and chemokines and direct killing. In addition to recognition of extracellular pathogens or foreign antigen, the innate immune compartment can recognize warning signals from within the cell or danger associated molecular patterns (DAMPs) and trigger inflammatory responses. For example, the NOD (nucleotide-binding oligomerization-domain protein)-like receptor NALP3 can recognize signals such as bacterial RNA and LPS. However, NALP3 can also recognize certain reactive oxygen species or other DAMPs which activate IL-1b via caspase-1, and in turn stimulate production of IL-6, thereby increasing the inflammatory environment[76]. The acute inflammation process, driven by mechanisms such as NALP3, recruits cells and soluble factors to restrict access to the site, eliminates the infectious agent and repairs damage to the local environment[77]. Tissues that are more likely to encounter pathogens are staffed with populations of immune cells that are poised to respond, such as DCs, which enact several functions including antigen capture, antigen presentation and cytokine/chemokine production. One subset of DC, the plasmacytoid DC (pDC), are specialized to produce copious amounts of type I interferons when challenged with various viruses[78]. Interestingly, interferons are classically defined as viral inhibiting cytokines but are associated with both anti-inflammatory and pro-inflammatory conditions[79]. Another cell of importance in the innate immune response is the NK cell, which distinguishes normal and altered conditions through a multiplexed system of activating and inhibitory receptors. The innate immunity portion of this thesis will focus on the NK cell phenotype and function in healthy and HIV-1 chronically infected Ugandans.

2.3 NK cells, receptors and function

NK cells were first reported in 1975 by two independent research groups working on cancer, where normal murine lymphoid cells were able to kill syngeneic as well as

allogeneic tumors from mice without prior sensitization[80-83]. This represented a major paradigm shift as effector lymphocytes were presumed to be T cells and operate by antigen exposure and recognition in a highly specific manner. In fact, early data was initially thought to be experimental artifact, and natural cytotoxicity was dismissed by some as merely noise in the chromium release assay used to measure suppressor T cell responses[84]. Despite the initial skepticism, NK cells have since been shown to be a significant population of large and granular effector cells, numbering up to 15% of circulating lymphocytes, with a wide range of functions and utility. Approximately 2 billion NK cells are circulating throughout the body at any given time, descending from a common lymphoid progenitor cell and ultimately differentiating from CD34+ hematopoetic stem cells in the bone marrow[85]. It is unclear where NK cell differentiation occurs, as NK progenitors have been isolated from the bone marrow and thymus, but there appears to be a requirement for proliferative cytokines as these NK cell precursors express CD122, the common β chain for IL-2 and IL-15 and require these cytokines to differentiate *in vitro*[86,87]. NK cell precursors can be found in the secondary lymphoid tissues, such as lymph nodes and mucosal associated lymphoid tissues (MALT), where cells identified as CD34+ CD45RA+ NK cell precursors were found in areas associated with DCs expressing high amounts of membrane bound IL-15[85]. While our understanding of NK cell development is not complete, the presence of these cells in a wide range of tissues implicates their importance in a number of immune functions. In addition, a number of clinical cases associated with an aberrant NK cell compartment have shed insight into the role of NK cells in disease as well as identifying some of the critical pathways of a cytotoxic NK cell[88]. While not common, NK cell deficiencies in humans are associated with immune dysfunction and lack of control of bacterial, fungal and viral pathogens in particular herpes viruses that in several cases prove fatal[88]. While many of the genetic or acquired conditions that result in NK cell loss or loss of function are not unique to NK cells, it is clear that these cells are a central component to the human immune system.

Since the discovery of NK cells over 35 years ago, a growing body of data is accumulating regarding the complexity of how these cells detect danger or stress. In 1985 and 1986, two revolutionary papers redefined basic immunology principles by proposing that NK cells might recognize or sense missing major histocompatibility complex (MHC) molecules on the surface of cells as an alternative immune strategy[89,90]. In 1981, Klas Kärre, a doctoral student at the Karolinska Institutet, originally proposed this theory later called the “missing self” hypothesis in the final chapter of his PhD thesis[91]. We now understand that viruses and tumors have evolved mechanisms to evade the adaptive T cell immune response by reducing the level of MHC molecules on the surface of cells in order to escape detection. NK cells are able to sense the altered expression of MHC and kill those cells through a number of receptors designed to monitor human leukocyte antigen (HLA), in addition to a number of other receptors that regulate their function. The NK cell array of stimulatory and inhibitory receptors includes: killer cell immunoglobulin-like receptors (KIRs), C-type lectin receptors, natural cytotoxicity receptors (NCRs), and TLRs[92,93] (Figure 4). As a matter of convention and lack of NK cell specific or universal NK markers, two cellular markers are used to identify and characterize human NK cells. CD56 is the neural cell adhesion

molecule (NCAM) and CD16 is the Fcγ-receptor IIIa, which is the low affinity binding receptor that recognizes the Fc portion of IgG antibodies. CD56 and CD16 flow cytometric staining intensities segregate NK cells into two functional subsets, immunomodulatory and cytotoxic with $CD56^{bright}CD16^{+/-}$ representing the former and $CD56^{dim}CD16^{+/-}$ representing the later and most substantial subset (up to 90% of NK cells)[85,94,95]. A third subset characterized as $CD56^{neg}CD16^{+}$ has also been described in the literature[96] and will be discussed later in this thesis. It is important to mention that the receptors found on the various subsets of NK cells are also found on other lymphocyte lineages and only the NCRs are thought to be restricted to NK cells.

The KIRs represent a group of activating and inhibitory receptors that may regulate the immune response to pathogens or cellular transformations. There are 17 *KIR* genes coding for 9 inhibitory receptors, 6 activating receptors, and 2 pseudogenes that are not expressed[97,98]. Over 30 KIR haplotypes exist that can be divided into groups based on absence (haplotype A), or presence (haplotype B) of activating KIRs[99]. Several MHC class I molecules are ligands for certain KIRs and a growing interest has developed surrounding these interactions, because *KIR* and *HLA* genes are highly polymorphic and certain KIR-HLA interactions may influence disease outcomes[100]. KIR3DL1, and probably also KIR3DS1, recognize HLA Bw4 allotypes with the nonpolar amino acid isoleucine (Bw4-80I), and to a lesser extent the polar amino acid threonine, at position 77-80 (Bw4-80T)[95,101]. East African populations have low frequencies of the *KIR3DS1* allele and high frequencies of *KIR3DL1* alleles and HLA-B with the Bw4 motif, particularly with an isoleucine at position 80, compared to other populations globally[99]. Similarly, the inhibitory *KIR2DL2* and *KIR2DL3* gene-products are alleles of the same locus and recognize HLA-C group C1 molecules. They show a more balanced distribution, but favor KIR2DL3 expression in East Africa. The *KIR2DL1* gene is constitutively expressed across all populations, and the receptor it codes for recognizes HLA-C group C2 molecules[99]. Expression of KIRs is genetically controlled[102], and the role of self-MHC molecules in NK cell KIR repertoire formation is controversial[103,104].

In addition to the KIR repertoire, NK cells have a number of receptors that help activate and regulate the functional response such as NKp30, NKp44 and NKp46. NKp30 and NKp46 are constitutively expressed on NK cells, however NKp44 requires IL-2 activation to be upregulated[94]. The NCR ligands are insufficiently characterized. CD161 is a C-type lectin-like receptor with numerous activating and inhibitory genes in the mouse, however there is just one gene in humans with conflicting reports of inhibitory function[85] and activating function[94]. Others report the lack of the classic immunoreceptor tyrosine-based inhibitory motif (ITIM) or charged amino acids necessary to transmit an inhibitory or activating signal respectively[105]. The ligand for CD161 is a non-MHC lectin-like transcript-1[85]. NKG2D is a well characterized activating C-type lectin and responds to cellular stress due to infection or transformation by upregulation of stress ligands such as MICA, MICB, and ULBP1-4[94]. Another group of receptors on the surface of NK cells that are type II C-type lectin-like membrane proteins are the NKG2 receptors and include NKG2A, NKG2C, NKG2E and NKG2F that form heterodimers on the surface of the cell with CD94[105]. *NKG2A* and *NKG2C* are the genes for the inhibitory and activating form of this receptor that recognizes HLA-E, a

non-classical MHC molecule, that binds the leader sequence from classic MHC and represents an indirect way in which NKG2A and NKG2C can monitor the expression of HLA-A, -B and –C molecules on the surface of the cell[94]. NKG2A is associated with a more immature NK cell phenotype that is purported to have less cytotoxic potential while NKG2C, the activating form, is more prevalent on cytotoxic NK cells[94],however expression may be stochastic[105]. There are a number of additional receptors that influence NK cell response but are not covered in this thesis.

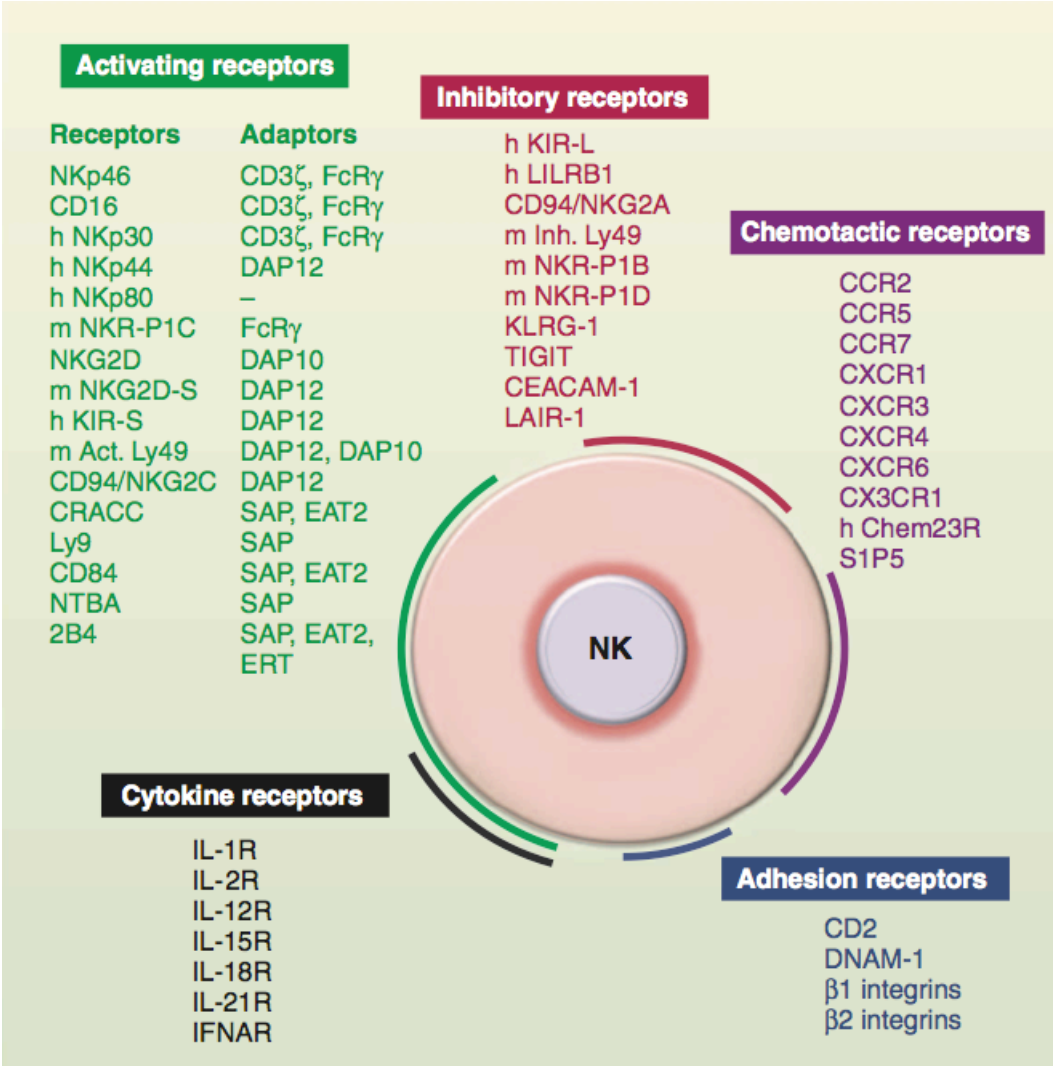

Figure 4. NK Cell Receptors (adapted from Vivier, E., *et al.*, 2011)[106].

As NK cells mature, they lose intensity of CD56 expression and gain CD16 expression along with increased cytotoxic potential. These CD56dim NK cells have less inhibitory NKG2A and are fully mature effectors cells with their full complement of KIR receptors. Utilizing expressed receptors and in conditions where activating signals outweigh inhibitory signals, NK cells are able to recognize and kill infected or malignant cells [91-93]. NK cells are able to lyse target cells through multiple mechanisms including transfer of cytotoxic granules through the immunologic synapse, Fas ligand (FasL) mediated

apoptosis, and antibody dependent cellular cytotoxicity (ADCC)[107]. Our recognition of ADCC pre-dates that of NK cells with early observations of "non-immune" lymphocytes selectively killing targets in the presence of target specific antibodies[108,109]. In 1987, research in the HIV-1 field showed that NK cells could recognize virus infected cells specifically through ADCC[109]. Interestingly, this innate and adaptive partnership gives FcγR bearing NK cells an adaptive feature of antigen specific recognition. NK cells are also able to directly kill transformed or infected cells with specialized cytotoxic granules containing perforin and granzyme, a process known as natural cytotoxicity. The cytolytic process requires cell adhesion and activation followed by an intracellular reorganization and facilitated movement of the lytic granule to the immunologic synapse, transfer of the granules contents and detachment resulting in the destruction of the target cell[110]. Interestingly, there is evidence to suggest that signaling through different activating receptors could lead to discrete molecular pathways involving enzymes involved in vesicle trafficking (eg. Rb27a), or proteins involved in fusion of cytotoxic granules to the cell membrane (eg. Munc13-4) that ultimately results in cell directed cytotoxicity[111]. The importance of granzyme- and perforin-dependent cytotoxic killing can be observed in inherited conditions such as Wiskott-Aldrich syndrome, where mutations in the *WASP* gene results in lack of WASP expression. WASP is critical in actin reorganization and formation of the immunologic synapse and lack of WASP results in severe immunodeficiency[110]. In addition to death mediated by cytotoxic granules, the death receptors such as FasL and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) are other mechanisms of NK effector function. TRAIL, also known as Apo2 ligand has been shown to be upregulated on NK cells in response to interferon and is associated with the clearance of certain tumor cells and response to viral infections[112]. FasL is stored intracellularly and can be sequestered to the surface through ligation of CD161 and has been shown to support the elimination of RMA-S tumor cells by perforin deficient murine NK cells[113,114]. Taken together, NK cells are lethal effectors with redundant mechanisms that allow for the control of transformed or infected cells.

NK cells also exercise their effector function via the production of cytokines and chemokines. $CD56^{bright}CD16^{-}$ NK cells are generally considered to be more immature with less KIR expression, more inhibitory NKG2A and are functionally skewed toward immunomodulatory cytokine production. The immature status of $CD56^{bright}CD16^{-}$ NK cells is supported by the fact these cells have longer telomere length, indicating fewer cellular divisions, and upon cytokine stimulation, these cells can differentiate into a more mature $CD56^{dim}$ NK cell[115]. $CD56^{bright}CD16^{-}$ NK cells express higher amounts of CD62L, a surface molecule allowing binding to the luminal surface of high endothelial venules (HEV) and further trafficking of NK cells out of the peripheral blood[116]. Furthermore, the chemokine receptor CCR7 is found on the $CD56^{bright}CD16^{-}$ subset at high levels, targeting them to lymph nodes[117]. These cells also express high levels of CCR5, CXCR3, and CXCR4[118]. Interestingly, $CD56^{bright}CD16^{-}$ NK cells are found in greater numbers in the secondary lymphoid tissue, constituting as much as 75% of the NK cells found in the lymph nodes, while approximately 15% are observed in the spleen and as few as 5% in the peripheral blood[119]. In addition to the location, $CD56^{bright}CD16^{-}$ NK cells are able to produce cytokines and chemokines more effectively than their $CD56^{dim}$ counterparts and are able to proliferate in response to low level cytokine stimulation[85].

Furthermore, this NK cell subset is found in secondary lymphoid regions that have a high concentration of DC and T cells whereby NK cells can respond and direct responses both through soluble factors and cell-to-cell contact[120,121]. In summary, $CD56^{bright}CD16^{-}$ NK cells are a unique NK cell subset preferentially capable of modulating immune responses in concert with DC and T cells in a non-cytotoxic fashion and subsequently influence the adaptive immune arm.

NK cells are classically considered an "innate" effector cell, but this definition may not be entirely accurate as more is learned about this lymphocyte subset. Adaptive immunity, which will be addressed in the next section, is understood to involve antigen encounter and specific recognition by T and B cells, followed by a period of proliferation and establishment of memory cells poised to act upon antigen re-exposure. Until recently, this was not considered a trait of NK cells. Antigen specificity has been demonstrated in murine NK cells through the activating Ly49H receptor in cytomegalovirus (CMV) infected mice, where a preferential expansion of Ly49H+ NK cells resembles the clonal expansion observed in T cells[122]. Furthermore, expanded Ly49H+ memory NK cells exhibit elevated function both with type 2 interferon (IFN)-γ production and degranulation measured by CD107a after ex vivo stimulation with antibodies against Ly49H or with cells expressing the CMV protein m157[123]. Originally demonstrated to be relatively short lived with a half life of 7-10 days[124], NK cells are now believed to differentiate into longer lived memory cells and in the case of the CMV Ly49H+ NK cells, residing in both lymphoid and non-lymphoid tissue that respond more readily to antigen re-exposure[123,125]. In addition to the CMV model, new evidence suggests that NK memory cells can be generated to a wide variety of antigens from influenza, HIV-1 and vesicular stomatitis virus in hepatic NK cells expressing CXCR6[126]. Human NK cells are able to discriminate MHC molecules bound with specific peptides through inhibitory KIR[127-131]. Further characterization of the crystal structure of HLA-Cw3 peptide complex with KIR2DL2 shows that direct contact takes place at position 7 and 8 of the peptide and is contingent upon the amino acid residue at position 8 of the peptide within the MHC class I binding groove[132]. Collectively, this data demonstrates that NK cells possess germline encoded antigen specificity and that certain populations of NK cells are able to proliferate and establish memory-like effector cells in order to respond to antigen re-exposure, thereby suggesting that these cells possess both innate and adaptive like qualities.

2.4 Adaptive immunity

The adaptive immune system consists of clonally expanded, "antigen-specific" cells that are able to respond to a highly specific pathogen sequence. In 1965, Max Cooper and colleagues characterized a model of two lymphocyte lineages mediating different arms of adaptive immunity from experiments on White Leghorn chickens studying the effects of removing the thymus, the bursa of Fabricius, neither organ or removal of both on one day old hatchlings[133]. Max Cooper's thymus dependent cells and bursa dependent cells were later defined as T cells and B cells originating from multipotent progenitor cells within the bone marrow, maturing in the thymus and bone marrow, respectively[134]. We now understand that the mediators of adaptive immunity are lymphocytes that undergo gene-rearrangement and clonal selection. In the evolutionary tree of life, adaptive

immunity appeared with the arrival of jawless vertebrates approximately 500 million years ago and is associated with assembly of variable immunoglobulin lymphocyte receptors[135]. However, vital components of the adaptive immune system are present in invertebrates (reviewed in [136]). In fact, homologs to recombination-activating gene (RAG)1 and RAG2, which mediate the gene-rearrangement necessary for the generation of B and T cell receptors, can be found in purple sea urchins (*Strongylocentrotus purpuratus*), although the function in these lower invertebrates remains unclear[137]. Through millions of years of evolution, adaptive cells have developed functions that overlap, complement and support innate immunity. These highly specific adaptive lymphocytes upon recognition of their cognate antigen, respond by proliferation, production of cytokines or chemokines, direct killing in the case of T cells and production of antibodies in the case of B cells. The complexity of antibodies and other soluble serum factors make up the humoral immune response, but the remainder of this thesis will focus only on the functions and phenotype of T cells.

Thymus-dependent cells (T cells) are the effectors of cell-mediated adaptive immunity and have a broad range of functions. The classical model suggests that T cells develop from multipotent stem cells giving rise to hematopoetic stem cells, the common lymphoid progenitor cell, and eventual development of T cell progenitors released from the bone marrow to the thymus where T cells develop and mature although the exact nature of this process continues to be explored[87,138]. Not all T cells are dependent on the thymus for development as there is evidence for other T cell populations developing in the gut, such as murine intraepithelial T lymphocytes, but these cells may serve different functions and mature with different receptor arrangements[139]. T cell progenitors that migrate into the thymus undergo a highly discriminatory process of clonal selection and deletion where less than 5% fully mature and migrate out of the thymus[140]. It is within the thymic environment of cytokines, chemokines and stromal cells that T cell gene rearrangement, differentiation and T cell selection can occur. T cell progenitors lack expression of the molecules CD3 (the proxy marker of T cells), CD4+ (helper T cells) and CD8+ (cytotoxic T lymphocytes, CTL). As the progenitor cells become committed to T cell differentiation, the process of T cell receptor (TCR) gene-rearrangement takes place starting with the β chain V, D, and J segments followed by proliferation, expression of both CD4 and CD8, and finally rearrangement of the α chain V and J gene segments resulting in the formation of the TCR [141,142]. In addition to the αβ chain TCR, the γδ T cell results from the Vγ and Vδ chain rearrangement, however this population is far less frequent, does not go through the rigorous selection as does the αβ T cell within the thymus and possesses a markedly different functional and phenotypic profile that will not be reviewed in this thesis. The αβ gene rearrangement, from the widely diverse V, D, and J segments, gives each progenitor a highly specific TCR. The total diversity of all TCR clones is on the order of 10^{18} resulting in an adaptive immune system with the ability to recognize a wide variety of antigens[75]. The CD4+CD8+ αβ TCR+ T cell progenitor goes through a complex selection process based on receptor binding strength to MHC-peptide complexes, intracellular signaling through molecules such as lck and zap70, and in an IL-7 dependent down selection of either CD4 or CD8 receptor completing development[143,144]. These antigen naïve CD4+ T cells and CD8+ T cells emigrate out of the thymus ready to survey circulation for antigen.

Antigens derived from intracellular and extracellular material are presented to T cells through MHC class I and class II molecules, respectively. MHC class I molecules present small peptides, 8-10 amino acids in length, to CD8+ T cells for a cell-mediated immune response that includes cytokine/chemokine production and direct cytotoxic killing. Intracellular antigens, both host and foreign, are screened to monitor infection or altered self. Processing and presentation include proteolysis, folding and stabilization of the class I molecule, loading of peptide fragments into the MHC complex, followed by transport to the cell surface. Proteolysis involves targeting proteins, via ubiquitination, for degradation by the proteasome. The proteasome is made up of the 20S tube subunit and capped by 19S subunits on both ends (reviewed in [145]). Proteins are tagged, unfolded and inserted into the inside of the 20S cylinder where the catalytic activity cuts the proteins in to small peptides. TAP1 and TAP2 are heterodimer complexes that actively transport these peptide subunits from the cytosol to the lumen of the ER[146]. Once inside the ER, peptides are loaded into MHC class I molecules and peptide-MHC complexes are then released from the TAP-tapasin complex and transported to the surface of the cell for presentation[147]. Alternatively, MHC class II molecules present peptides >12 amino acids in length to CD4+ T cells. Unlike the MHC class I presentation pathway, MHC class II antigens are classically considered to be exogenous material including foreign pathogens. Antigen uptake is achieved through a number of mechanisms such as macropinocytosis and phagocytosis. Antigens are taken up into endocytic vesicles that undergo maturation through which certain reactive oxygen species are generated, pH is decreased by proton pumps, and activation of proteases allows for the degradation of antigen into peptide fragments[148]. Mature endocytic vesicles containing processed antigen fuse with MHC class II loaded vesicles where peptides replace the invariant chain peptide and bind into the MHC class II groove[149]. Interestingly, additional proteolytic processing of the peptide can occur after the peptide is loaded into the MHC class II molecule but does not alter the amino acids contained within the groove[150]. These peptide loaded MHC class II molecules are transported to the cell surface and present the peptide to CD4+ T cells.

HLA is the system of MHC molecules in humans and the genes that code for these molecules can be found on human chromosome 6. The human MHC class I antigens include HLA-A, HLA-B and HLA-C, while human MHC class II antigens include HLA-DR, HLA-DP, and HLA-DQ. There is extreme allelic diversity in the genes that make up the HLA system globally. For HLA class I there are 1519 HLA-A, 2069 HLA-B and 1016 HLA-C alleles, and for HLA class II there are 969 HLA-DR, 179 HLA-DQ, and 173 HLA-DR alleles (from the IMGTT/HLA online database)[151]. The HLA class II molecules are more complicated because there are two chains, the α and β chains making up the functional portion of the heterodimeric peptide binding protein, whereas the class I HLA functional portion is derived from only the α chain. In addition to the major class I antigens, there are three minor HLA antigens; HLA-E with 10 alleles, HLA-F with 22 alleles, and HLA-G with 46 alleles known[151]. The enormous diversity of both the HLA class I and II alleles results in a varying ability to bind different peptides into the groove of the MHC molecule. In fact, the highest degree of variability in the MHC region occurs at sites that affect peptide binding into the MHC molecule[152]. This may account for differential ability among populations in protection from certain diseases. African

populations possess the greatest diversity in the MHC region, particularly HLA class I molecules[152].The polymorphic system of MHC class I and II in humans presents a wide range of peptides derived from "self" and "non-self" to the cells of the immune system in order to direct an appropriate response.

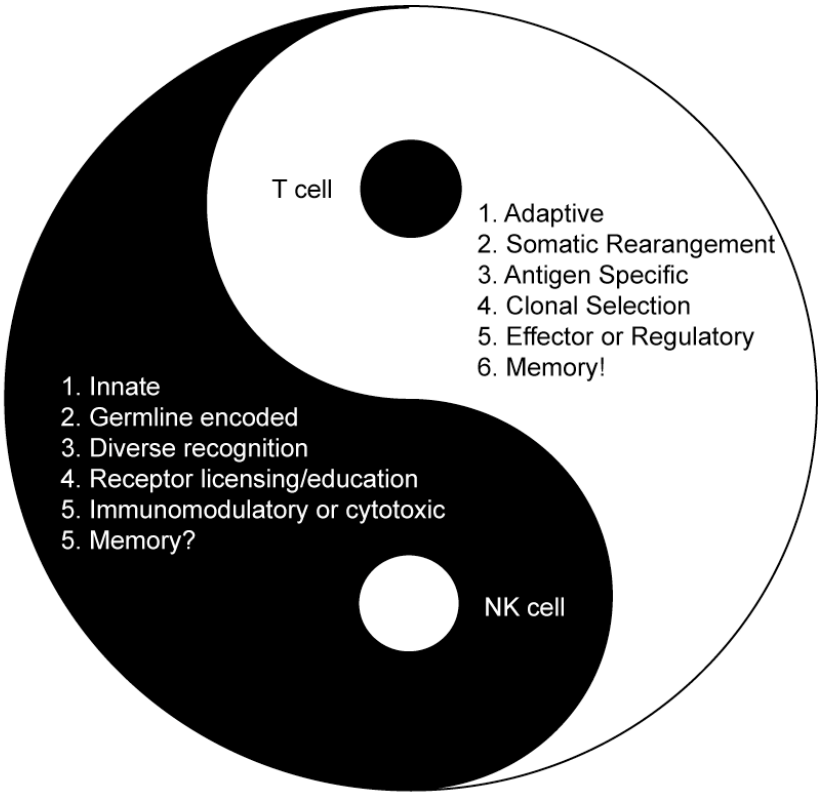

Figure 5. The yin and yang of cell mediated immunity.

Signaling of T cells through their TCR by HLA presented peptides stimulates a wide array of functions such as cytotoxicity, immune modulation and regulation. In normal healthy conditions, CD4+ helper T cells outnumber CD8+ cytotoxic T cells approximately 2:1 in peripheral blood circulation. CD4+ T cells play a more dynamic role in the immune system as they are integral in supporting B cells to make antibodies, enhance CTL killer function, regulate and suppress aberrant immune responses, and communicate with a number of cells in the immune system to coordinate efficient responses[153]. Originally, CD4 cells were proposed to direct a bimodal immune response as shown in mice producing cytokines in responses to a number of antigens such as fowl g globulin and chicken red blood cells, where type 1 (T_H1) was associated with IL-2 and IFN-γ and type 2(T_H2) was associated with B cell stimulating factor 1 and T cell growth factor 2 (now known as IL-4)[154]. In fact, the cytokine response that corresponds with a T_H1 lineage is considered to be involved in directing cellular activity while T_H2 helper cells could be more involved in humoral support. The heterogeneity of CD4+ T cells is now known to be more complicated with additional lineages identified such as T_h17, regulatory T cells (T_{reg}), and follicular helper T cells (T_{fh}) which all possess unique functional qualities, are found in certain tissues and have distinct phenotypes[153].

Furthermore, CD4+ T cells have been shown to effect cytotoxicity through granzyme and perforin release in an MHC class II dependent manner[155]. CD8+ T cells have a slightly different process of differentiation and function. In the presence of proper TCR stimulation and costimulation, a CD8+ T cell will turn on to produce cytokines and chemokines while killing target cells through a number of mechanisms including directed cytotoxic granules and death receptors[156,157]. Further classification of CD4+ T cell and CD8+ T cell distinct subsets can be made through examination of the diverse surface markers that correspond to different activation and maturational states. Some of these receptors will be reviewed in the next section.

2.5 T cell receptors and functions

Stimulation of naïve T cells requires a combination of signals, usually first involving ligation of the TCR with corresponding peptide loaded HLA molecule and followed by co-stimulatory signals through CD28 or other similar receptors found on the surface of the T cell in order to promote survival over apoptosis[158]. Some of the surface molecules involved in providing co-stimulation include: CD28, inducible T cell costimulator (ICOS)(CD278), lymphocyte function associated antigen 1 (LFA-1)(CD11a/CD18), signaling lymphocytic activation molecule (SLAM), 4-1BB (CD137), OX40 (CD134) and CD27 (all reviewed in [158-160]). In addition to co-stimulatory signals, receptors exist to negatively regulate immune responses through inhibitory signals such as cytotoxic T lymphocyte antigen-4 (CTLA-4)(CD152) and programmed death-1 (PD-1)(CD279)[159]. PD-1 is a 288 amino acid transmembrane protein with an ITIM and an immunoreceptor tyrosine-based switch motif (ITSM) intracellular domain[161]. This inhibitory member of the CD28 family can be expressed, especially during activation, on T cells, B cells, natural killer T cells and dendritic cells[161]. PD-L1 and PD-L2 engagement of PD-1 is thought to participate in balancing the immune response by down regulating activation. PD-1 co-inhibition, in the mouse model, is dependent upon the strength of the PD-L1 inhibitory signal, TCR engagement and CD28 co-stimulation[162]. Furthermore, CD28 co-stimulation and IL-2 can overcome the inhibitory signal provided by the PD-1 and PD-L1 interaction[162-164]. In other studies with primary human CD4+ T cells, PD-1 ligation overrides suboptimal CD28 co-stimulation resulting in decreased levels of IL-2, IL-10, IL-13, IFN-γ, and the anti-apoptotic protein BcL-xL production indicating not only compromised function but decreased cell survival[165]. The amount of inhibitory receptors varies on murine CD8+ T cells and it is proposed that many of these receptors are expressed in a non-redundant fashion suggesting that blocking the signal from one is sufficient to restore normal function[164]. Through this combination of signals that T cells receive, the dynamic nature of T cell responsiveness is formed.

In addition to co-stimulatory and co-inhibitory receptors, several molecules are found on the surface of T cells and are associated with an "activated" phenotype. CD69 is a c-type lectin found on the surface of recently activated lymphocytes, including T cells, and is associated with proliferation and Ca++ influx[166,167]. CD25 is the interleukin (IL)-2 α chain receptor and is up-regulated 12-24 hours after antigen activation and is associated with the generation of the high affinity IL-2 receptor, proliferation and differentiation[168]. Interestingly, a recent report by Geldmacher and colleagues examining co-infection of mycobacterium show that HIV-1 preferentially infects

stimulated CD4+ T cells expressing CD25 on their surface[169]. Two additional activation markers that are relevant in HIV-1 infection are CD38 and HLA-DR[170]. HLA-DR is a MHC class II molecule and has been shown to be up-regulated 48-60 hours after antigen stimulation[168]. HLA-DR is primarily found on immune cells and is up-regulated due to TCR stimulation in T cells or IL-4 exposure in B cells[171]. Experiments conducted *in vitro* show macrophage expression of HLA-DR can be down-regulated due to prostaglandin E2 while supernatant from mitogen activated T cells or purified IFN-γ will up-regulate class II molecules on macrophages and endothelial cells, which under normal conditions do not express class II molecules[172]. *In vivo* studies studying daily intraperitoneal IFN-γ administration in mice show elevated class II MHC expression widely across many tissues such as heart, kidney, liver, lung and spleen, however increases were not observed in lymph nodes[173]. Additional information is needed in order to elucidate the cause of increased expression of HLA-DR on T cells and the consequence of sustained periods of expression. CD38 is an ectoenzyme found ubiquitously on the surface of immune cells and has been associated with states of activation in T cells[174]. Originally identified in 1980, CD38 is now known to be involved in a number of functions including catabolism of ADP-ribose, NAD+, recruitment of Ca^{2+}, internalization of nucleotides and chemotaxis to endothelial cells expressing CD31 (reviewed in [175]). Hon Cheung Lee's identification of similarity in sequence to the ADP-ribosyl cyclase from the sea mollusk (genus *Aplysia*) allowed for the critical link to identify enzymatic activity and also marks the importance of this molecule as highly conserved through evolution where enzymatic homologs are found, predating humans by 700 million years[176,177]. The significance in T cell activation may come from the metabolic demand put on proliferating and functional cells responding to antigen. A switch from oxidative phosphorylation to aerobic glycolysis gives more products necessary for growth and survival[178]. In addition, CD38 has been associated with lateral co-localization of TCR, BCR and CD16 and may be involved with the metabolic requirements of their signaling[175]. Examining these markers of activation individually or in tandem may help characterize aberrant forms of activation associated with certain diseases and identify potential targets to mitigate the response.

Maturation or differentiation of T cells is the process that occurs in CD4+ naïve or CD8+ naïve cells after emigration from the thymus and in response to activation by their cognate antigen. This process is paired with proliferation and the generation of effector cells, effector memory cells, and long-lived central memory cells. The precise pathway that T cells take to become memory cells is not entirely understood, but a number of investigations in human disease and vaccine settings have characterized some of the homing and phenotypic profiles that define discrete populations of memory cells. Some of the markers used to identify effector and memory cell subsets include CD27, CD28, CD45RA, CD45RO, CD57, CD62L, CD127, and CCR7. CD45RA and CD45RO are isoforms of the protein tyrosine phosphatase receptor type C and are associated with naïve and effector populations on the CD45RA+ cells and memory populations from the CD45RO+ cells[179]. CD27 is a co-stimulatory member of the TNF-receptor family and engagement with its ligand, CD70, can enhance proliferation and cytokine production[160]. CD28 is another co-stimulatory molecule mentioned above and both CD27 and CD28 are associated with less antigen-experienced stages when expressed on the surface of T

cells. CD57 is a glycoprotein found on the surface of numerous cells, but on T cells has classically been associated with replicative senescence[180]. The IL-7 receptor α chain, CD127, is associated with a memory phenotype in CD8+ T cells specific for vaccinia virus[181]. Finally, markers to characterize the homing potential of T cells are commonly used to differentiate cells. CC chemokine receptor 7 (CCR7) and L-selectin (CD62L) are both surface receptors that allow trafficking through high endothelial venules and expression of these markers is associated with central memory and naïve phenotypes, but is poorly predictive of effector function[182]. In order to develop vaccines or other interventions that harness subsets of T cell immunity, more information is needed to elaborate the model of T cell differentiation and the generation of long-term memory cells.

The adaptive immune response has evolved mechanisms to protect the host from the deleterious effects of prolonged inflammation or self-directed responses. One method involves T cells with unique phenotype and function that have the ability to manipulate and regulate the immune response, maintaining immune tolerance and avoiding inappropriate immune activation and autoimmunity. These cells are known as regulatory T cells (T_{regs}). T_{regs} develop from T cell precursors in the thymus or can be induced under certain cytokine environments in the periphery. They can regulate effector T cell responses through production of TGF-β, IL-10, IL-35, granzymes, perforin and receptor mediated signaling (reviewed in [183]). Traditionally, T_{regs} are identified as CD4+, CD25+ and forkhead box protein 3 (FoxP3)+, however identification of these cells continues to be debated as other surface characteristics such as reduced CD127, the IL-7 receptor, may be a better predictor of suppressor function in human CD4+ T cells[184]. FoxP3 is a transcription factor that has a role in T_{reg} function, however in cooperation with other transcription factors may play an important role in proliferation, differentiation, and cell development[185,186]. A number of phenotypic and functional characteristics have been associated with discrete subsets of $T_{regs.}$ For example, T_{regs} that are targeted to the skin will express αE integrin chains, CCR4, E selectin and P selectin. Regulatory responses targeted to T_H1 effector T cells are through factors mediated by T-bet, a transcription factor shared by both the IFN-γ producing T cells and T_{regs}[183]. Additionally, T_{regs} are able to prevent adaptive immune responses through cross talk with dendritic cells, where T_{regs} expressing CTLA-4 are able to prevent expression of co-receptors CD80 and CD86 thereby preventing effective priming of a T cell response[187]. There are many more phenotypic markers that are associated with the function and location of T_{regs} indicating that these cells have oversight of many lymphoid and non-lymphoid compartments.

The functional heterogeneity of T cells continues to be explored in relation to location throughout the human body, cell phenotype and the functional profile exhibited in various disease conditions. In many viral infections, production of certain cytokines such as IFN-α, -β, -γ and TNF-α have direct anti-viral affects whereas other cytokines such as IL-1α, IL-1β, IL-2, IL-6, IL-12, IL-13 and IL-18 modulate immune responses indirectly[188]. T cells are potent sources of IFN-γ, TNF-α and IL-2. Chemokines are secreted proteins that are involved in the recruitment of immune cells to sites of inflammation and certain chemokines such as macrophage inflammatory protein

(MIP)–1α, MIP-1β and RANTES have been shown to reduce HIV-1 target cell entry through blocking of CCR5[189]. In addition to soluble factors, cell effector responses can result in clearance of virus through lysis of infected targets, which are mediated by cytotoxic granules containing perforin, granzyme A and granzyme B. One molecule that is associated with release of cytotoxic granules, aptly named degranulation, is lysosomal-associated membrane protein 1 (LAMP-1 or CD107a) found within lytic granules and is transiently expressed on the surface of cells upon granule release. This marker is commonly used to study both CD8+ T cell cytotoxicity[190] and NK cell cytotoxicity[191] during *in vitro* assays. Another measure of T cell function is proliferation in response to antigenic stimulation. Historically, proliferation was based upon radioactive assays to measure bulk proliferation. However, more recent flow based assays using carboxyfluorescein diacetate succinimidyl ester (CFSE) can allow for discrete determination of daughter generations as well as phenotypic characteristics of proliferating populations[192]. An additional marker that is expressed in cells that are proliferating is Ki67, which corresponds to a nuclear protein involved in regulating cell division and is not expressed in quiescent cells[193]. Technological advancement in flow cytometry and intracellular cytokine staining has increased our ability to characterize the frequency, immunophenotype, and functionality of antigen specific T cells. Multi-functionality is typically assessed by the simultaneous measurement of in IFN-γ, TNF-α, IL-2, MIP-1β and CD107a in T cells responding to synthetic peptide pools derived from viral proteins.

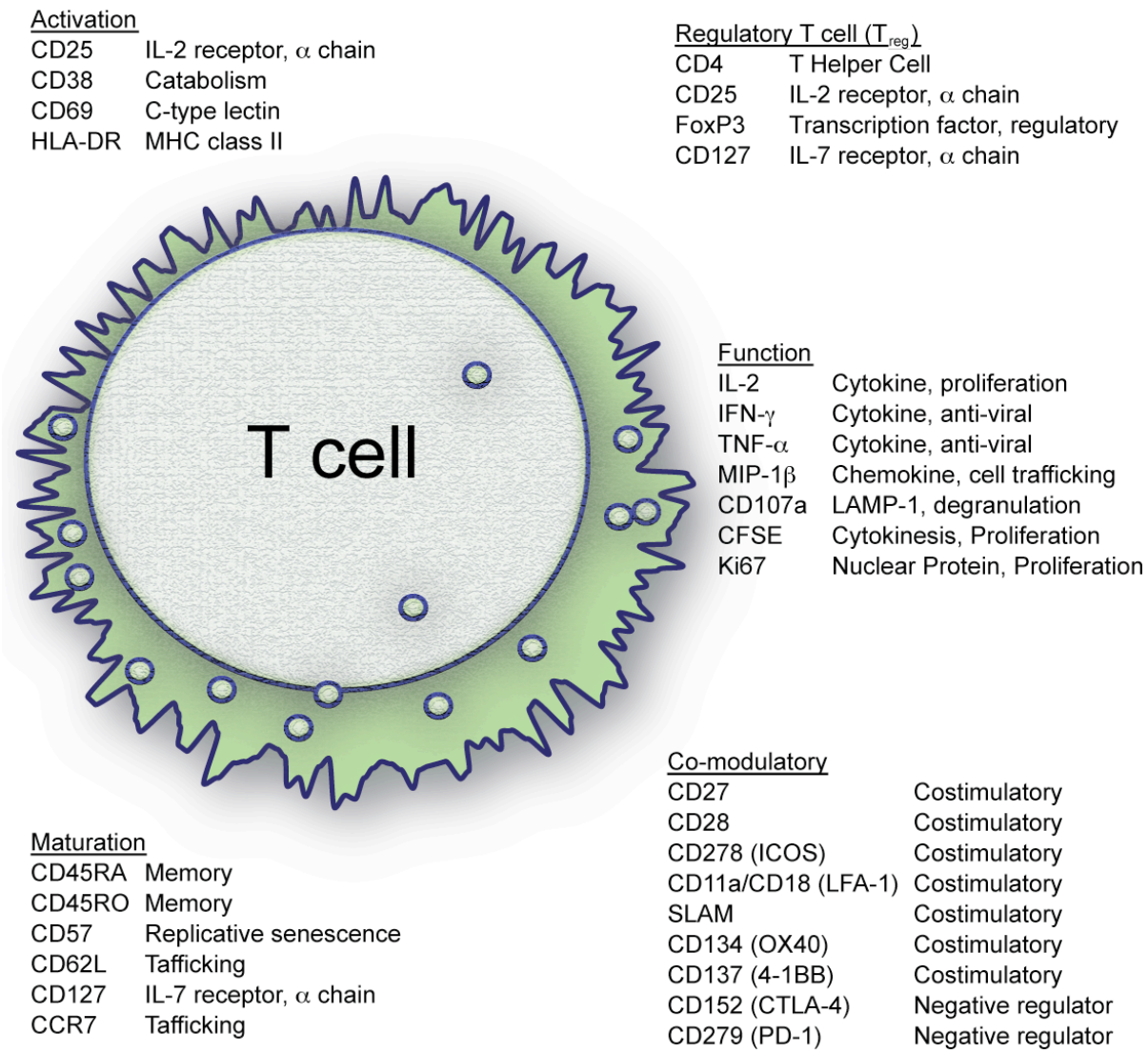

Figure 6. Common markers of T cell phenotype and function.

3. METHODS AND TECHNICAL NOTES

3.1 PBMC processing

In 1963, A. Böyum from the Norwegian Defense Research Establishment developed the first method for separation of peripheral blood mononuclear cells (PBMC)[194]. The process involved layering whole blood over fluid with a density of 1.077 g/ml (Ficoll-hypaque PLUS (Pharmacia, Uppsala, Sweden)) whereby after centrifugation, lymphocytes and monocytes were separated from plasma, red blood cells, and granulocytes[194]. This technique still proves to be the primary method of PBMC isolation used around the globe. One substantial development was the invention of conical tubes with a synthetic barrier, also called the frit, which allows for a more robust separation and is less sensitive to sudden movements or disruption of the plasma-ficoll interface. Commercially available tubes (such as Leucosep® Greiner Bio-One, Frickenhausen, Germany (a.k.a Accuspin tubes®) are generally used in order to maximize PBMC yields while reducing platelet and granulocyte contamination, although user technique is a critical component to effective PBMC processing as well. The method is outlined in Figure 7A. In short, whole blood is collected from venipuncture and sent to the laboratory for processing. The ACD anticoagulated blood is layered on top of preloaded Ficoll in Leucosep® tubes and centrifuged. The PBMC layer is harvested and washed with PBS before cryopreservation at a concentration of 10^7 cells/ml in freeze media and stored long-term in liquid nitrogen vapor at -140˚C.

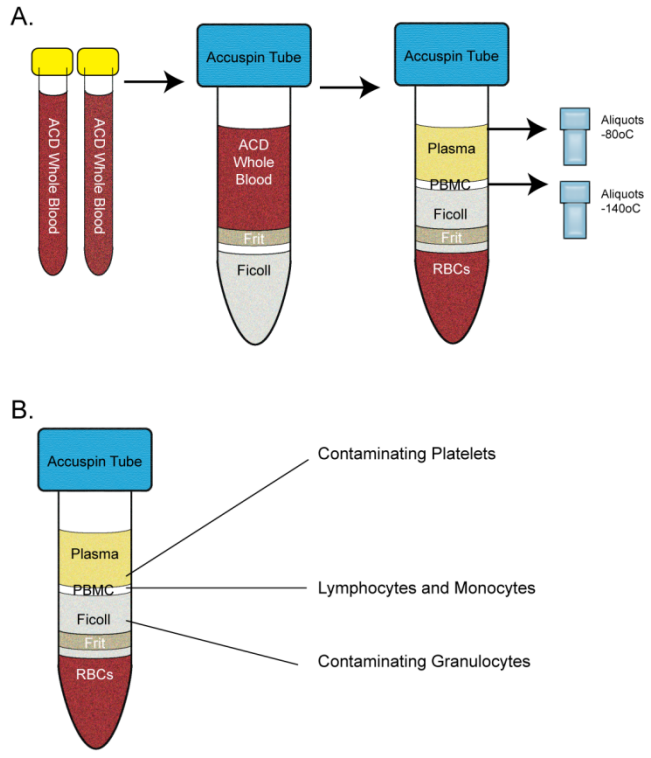

Figure 7. Overview of PBMC Processing.

3.2 Flow cytometry

The first flow cytometer patented in the US (US Patent 2,656,508)[195] was put forth by Wallace H. Coulter based upon a system that could quantify microscopic particles suspended in an electrolyte solution and measured by electrical impedance, a process known as the Coulter Principle[196]. Building upon the early counting chambers and the ability to distinguish simple characteristics of microscopic particle size was the ability to detect fluorescence. The Herzenberg lab at Stanford University was one of the first groups to successfully discriminate and sort cells (mouse splenocytes from Chinese hamster ovary cells) based on the intracellular expression of fluorescein and subsequent light emission after excitation with a blue laser[197]. These pivotal experiments led to the phrase fluorescence-activated cell sorting (FACS), which to many is synonymous with flow cytometry and remains part of the name of several Becton Dickinson BioSciences flow cytometry instruments. Second generation FACS instruments, in the 1980's and into the 90's progressed from measurement of size (light forward scatter), granularity (light side scatter) and single fluorescence channel to 3- and 4-color flow cytometry using 1 and 2 lasers, respectively. The ability to measure multiple parameters at the single cell level helped immunologists develop a better understanding of the complex nature of lymphocyte phenotype and function, particularly in T cell characterization. Toward the end of the 1990's and into the 21st century, a rapid expansion in technology and reagents has witnessed 11-color[198,199] and up to 17-color flow cytometry[200]. Moreover, the field of multi-parameter flow cytometry beyond five to six colors has been termed polychromatic flow cytometry (PFC)[201], and many labs have developed this capacity with a wide range of applications.

It is important to review the underlying technology of current flow cytometry in order to better understand the hurdles in PFC. Immunofluorescently labeled cells that have typically been fixed and are in a buffered saline solution are acquired into the fluidics system of the flow cytometer where they are funneled into the flow cell. In theory, a single cell line passes through the flow cell where up to three or four laser beams of varying intensity intersect and excite specific fluorescent markers on each cell, thereby emitting various wavelengths of light. The light emissions are detected by photomultiplier tubes (PMT), then translated and recorded as voltage pulses. These pulses are then converted in to electrical signals, amplified and finally stored into the computer software for real-time or batched analysis. Each laser is designed to stimulate specific fluorochromes that have a range of spectral emission requiring coordination with specific PMTs. One of the major advancements in flow was the trigon and octagon orientation of the multiplier tube detector array in order to maximize different parts of the light spectrum. For example, the 633 nm red laser hits certain fluorochromes which each have a spectral range. The orientation of the trigon detector array (corresponding to the ability to detect up to three fluorescent signals) allows for the incoming light from the flow cell to be focused to the first PMT, "A". In front of the PMTs sit a longpass filter and a bandpass filter. The longpass is rated at a specific spectral wavelength and allows all light of higher wavelength to pass while deflecting the light at lower wavelength onto the second PMT "B". The longpass filter in front of "B" passes lower wavelength light onto the next PMT "C" and in the case of the octagon detector array goes on to up to eight potential PMTs. The band pass filter is rated at a specific wavelength of a defined range and allows light within that range into the PMT while deflecting the remaining

light away. The detector is designed to go from higher to lower wavelengths in light. It is imperative when designing PFC panels to ensure the instrumentation is set up accurately in order to measure the specific reagents being used to fluorescently label cells. A detailed configurational layout of the flow cytometers used in this thesis has been displayed in Figures 8 and 9 for the BD LSRII and BD FACSCantoII respectively.

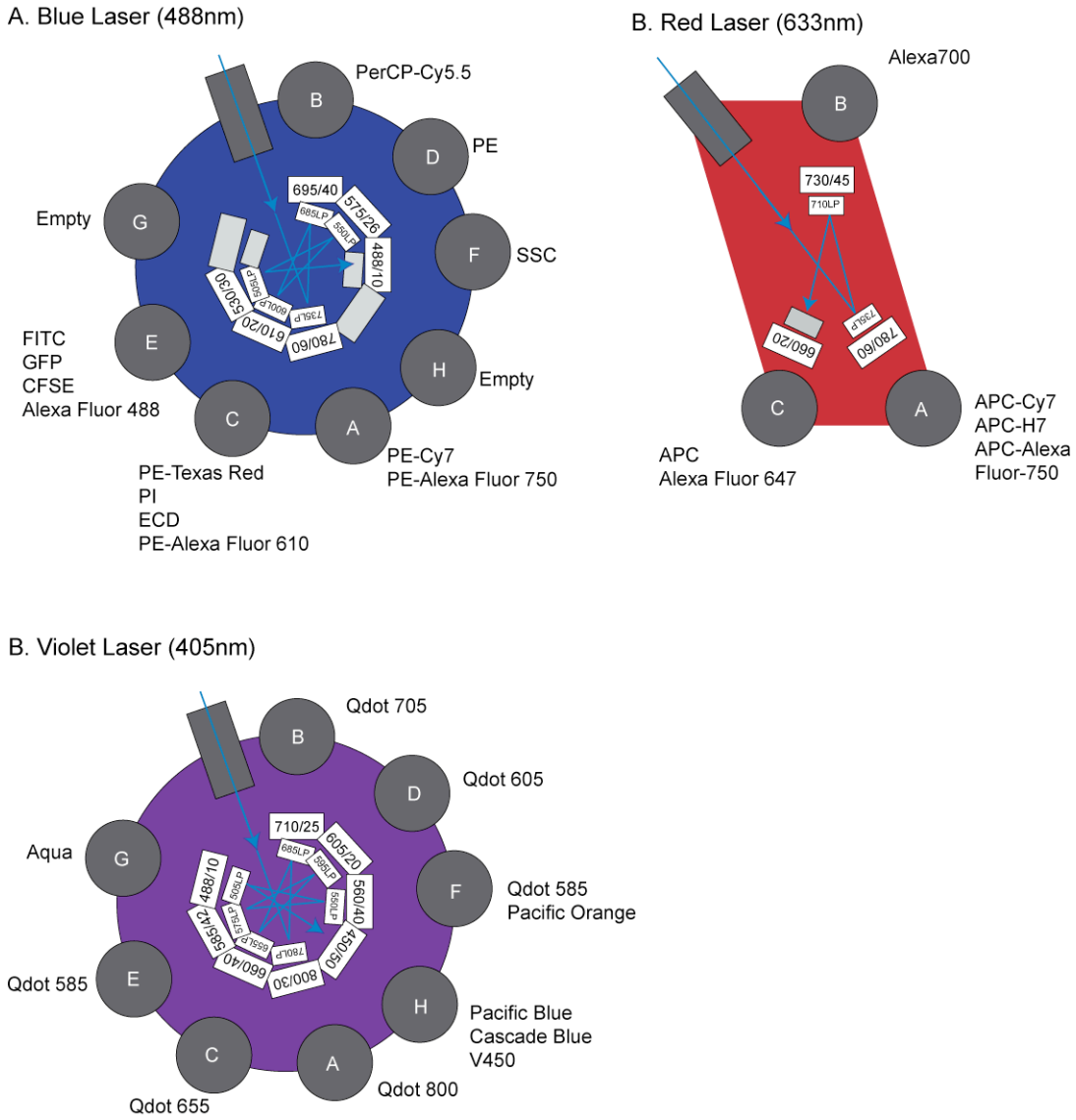

Figure 8. US - BD LSRII (3 laser configuration) used in PAPER II and PAPER IV.

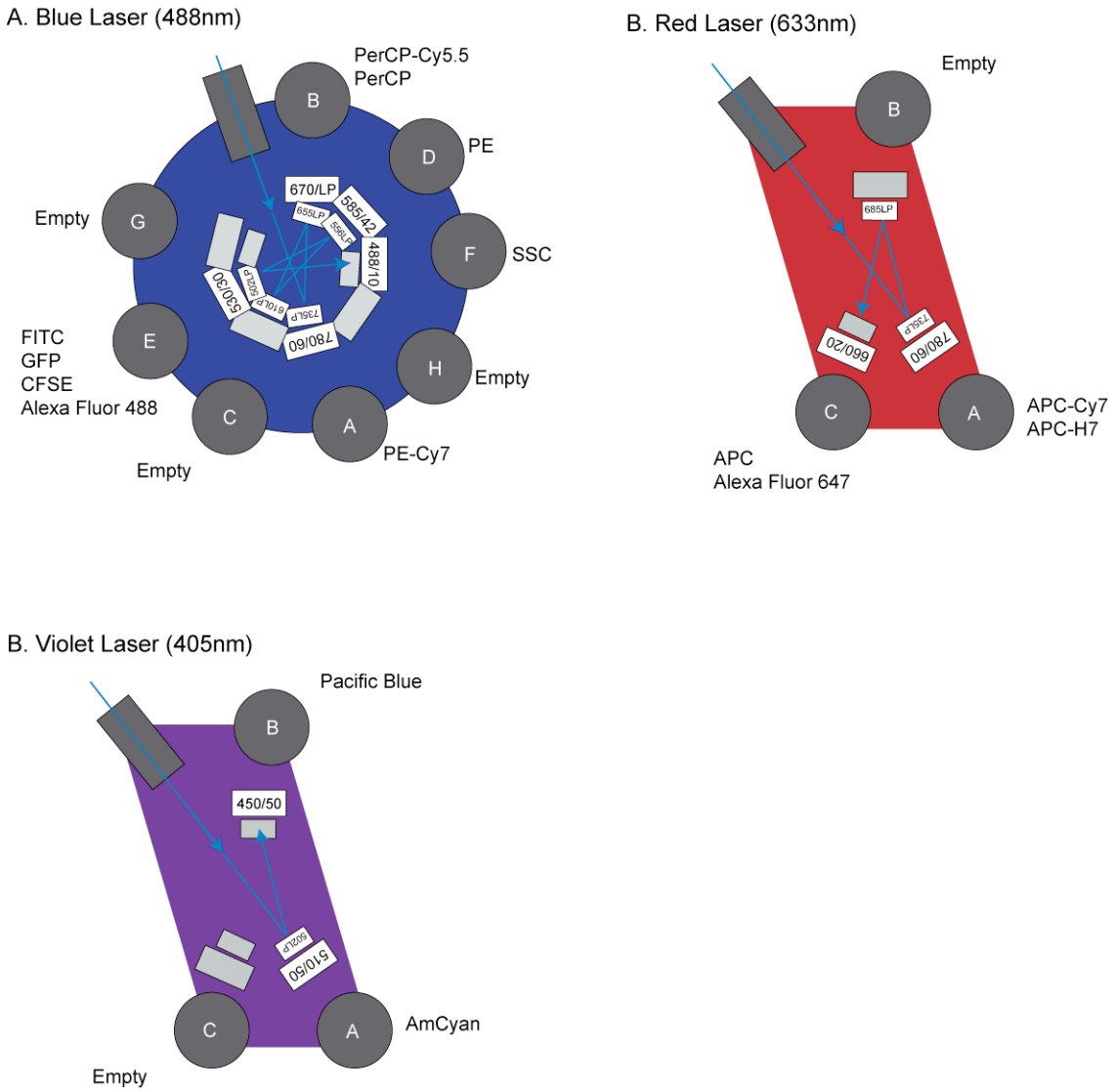

Figure 9. Uganda BD FACS Canto II (3 laser configuration) used in PAPER III.

One of the major challenges in leveraging the sensitivity over the specificity in flow cytometry is the ability to correct for spectral overlap between different fluorochrome emissions, a daunting task known as compensation. In the silver age of compensation, this was a manual process that was done before sample acquisition and involved looking at individually stained fluorochromes bleeding over into other channels and subtracting out the "false positive" emission to levels at or below auto fluorescence. This was quite manageable at three- and four-color instances of acquisition and analysis, however when going beyond this number of fluorescent analytes, manual compensation becomes impossible. Software has been developed to make this analysis possible and can be done before or after sample acquisition. PFC compensation is not without limitations and classic flow analysis strategies need to be reconsidered, such as classic quadrant gating which may no longer accurately segregate discreet populations uniformly[202]. Moreover, newer ways to visualize data can enhance data analysis and

interpretation and new scaling ("logical") allows for complete view of all populations[203,204].

The boom in technology and equipment for PFC occurred simultaneous to widespread availability of new antibody, fluorochrome and flow cytometry support reagents. One major discovery harnessed the power of semiconductor nanoparticles known as quantum dots or Qdots, which are inorganic crystals of cadmium selenide of various sizes that emit different spectral wavelengths[205]. Qdots utilize a number of fluorescence channels particularly when using the 405nm violet laser. Another important development in PFC was the ability to use fluorescence channels to discard unwanted populations as opposed to positively selecting required populations. As more flurochromes were added to flow panels, immunologists developed channels known as "dump channels" to accommodate the labeling of cells to exclude from a particular analysis. A common problem, particularly in indentifying dim populations at very low frequency, were spurious results often observed from auto fluorescence of dead cells. Amine-reactive dyes such as Aqua Live/Dead, utilized in this thesis, were developed in order to exclude dead cells from analysis and add an extra layer of quality built into flow analysis[206]. Commercially available synthetic beads coated with antibodies recognizing the Fc portion of human monoclonal antibodies used in flow cytometry were developed for the purpose of computing compensation matrices without wasting precious samples. Tandem dyes, or combinations of fluorochromes, have been designed to offer the immunologist more flexibility to utilize as many fluorescent channels as possible. In addition, new fluorochromes with reduced excitation ranges are becoming more widely available, creating less spectral overlap between reagents. Despite all the advances, PFC is still a relatively new technique and availability of monoclonal antibodies directly conjugated to rare fluorochromes are extremely limited and lot to lot variation remains problematic for consistent panel performance. In summary, careful optimization of newly designed panels and continuous evaluation of performance is critical for consistent and accurate PFC. A detailed list of the panels used in this thesis can be found in Table 1 and the actual antibodies used can be found in each paper.

Table 1. Primary flow panels used in published thesis research.

Laser	Fluorochrome	NK Phenotype 1	NK Phenotype 2	NK Phenotype 3	NK Phenotype 4	NK Function 1	NK Function 2	Isotype	T cell Activation	Treg
Blue 488nm	FITC	KIR2DL1	CD94	KIR3DL1	NKp46	CD107a	KIR3DL1	Isotype	HLA-DR	FoxP3
	PE	KIR2DL2/DS2/DL3	NKG2C	KIR3DL1/DS1	NKp44	MIP-1β	MIP-1β	Isotype	PD-1	CD25
	PE-Texas Red/ECD	CD3	CD3	CD3	CD3	CD3	CD3			
	PerCP-Cy5.5							CD3	CD3	CD3
	PE-Cy7	CD56	CD56	CD56	CD56	CD56	CD56	CD8	CD8	
Violet 405nm	PacB/V450	CD16	CD16	CD16	CD16	CD16	CD16	CD4	CD4	CD4
	Aqua	Live/Dead	Live/Dead	Live/Dead	Live/Dead	Live/Dead	Live/Dead	Live/Dead	Live/Dead	Live/Dead
	Qd 605	CD4	CD4	CD4	CD4	CD4	CD4			
Red 633nm	APC/AF647	KIR2DL3	NKG2A	CD161	NKp30	IFN-γ	IFN-γ	Isotype	CD38	CD127
	APC-H7	CD14/CD19	CD14/CD19	CD14/CD19	CD14/CD19	CD14/CD19	CD14/CD19			
Papers used in:		**PAPER II and IV**	**PAPER II**	**PAPER II and IV**	**PAPER II**	**PAPER II and IV**	**PAPER IV**	**PAPER III**	**PAPER III**	**PAPER III**
Cytometer used:		**BD LSRII (3 laser configuration)**						**BD FACS Canto II (3 laser configuration)**		

4. AIMS OF THESIS

The general aim of this thesis was to examine the role of cellular immune functions in chronic, untreated HIV-1 infection in Uganda. Originally, we sought to investigate phenotypic and functional qualities of T cells responding to HIV-1 in relation to rates of disease progression in natural HIV-1 infection. We were also interested in the frequency, function, and phenotype of NK cells in acute and chronic HIV-1 infection in our Ugandan cohorts. The data on NK cells would be anlayzed in relation to the immune responses detected and potential associations with viral subtype and disease progression determined. We also intended to investigate T_{regs} in acute and chronic HIV-1 infection. Original plans to examine acute infection prospectively in a cohort of individuals at higher risk to acquire HIV-1 in Uganda were not pursued due to protocol delays. Through the conduct of these cohort activities our specific study objectives were developed as follows:

Study Objective 1: PBMC are increasingly being used to monitor cellular immune functions in infectious disease and to assess vaccine efficacy. We established the capacity to process and cryopreserve PBMC in the resource limited setting, and aimed to define parameters to ensure the quality of product in HIV-1 infected and HIV-1 uninfected participants. **(PAPER I)**

Study Objective 2: NK cells are important innate cells that are involved in the first line of defense against viral infections. However, little was known about these cells in Ugandans. We aimed to characterize the phenotype and function of NK cells and subsets in HIV-1 chronic infection and search for possible correlates of viral control in HIV-1 subtype A and D infection. **(PAPER II)**

Study Objective 3: Chronic immune activation, especially in CD8+ T cells, is believed to be an important component of HIV-1 pathogenesis and predictor of disease progression. Immune exhaustion has also been proposed as an important factor in HIV-1 associated immune dysfunction. We aimed to investigate pathological CD4 T cell activation and exhaustion in infected Ugandans, with a focus on the potential driving forces behind persistent immune activation. **(PAPER III)**

Study Objective 4: Certain KIR and HLA combinations have been associated with favorable outcomes in HIV-1 infection by genetic epidemiological analysis. We aimed to characterize the role of inhibitory KIR and their cognate class I ligands in shaping the repertoire and function of NK cells in HIV-1 infected and uninfected Ugandans. Furthermore, we aimed to analyze these parameters in relation to disease progression. **(PAPER IV)**

5. RESULTS AND DISCUSSION

HIV-1 infection in Uganda represents not only a major medical crisis but poses severe socioeconomic implications that hinder many aspects of the country's growth and development. Major improvements to the health infrastructure have resulted in access to ART by many who are HIV-1 infected, but too many challenges remain to achieve universal access. Since the initial observations of HIV-1 over three decades ago, much has been learned about the virus and host interaction, yet immune control of HIV-1 is incompletely understood and more information is required for development of a globally efficacious HIV-1 vaccine. Through the aims of this thesis, information regarding cellular immunology in chronic untreated infection with HIV-1 subtype A and subtype D was obtained and presented in the attached manuscripts. In this results and discussion section, major findings will be put into context of the current epidemic in Uganda. This section is divided into smaller sections discussing the importance of processing and PBMC cryopreservation, NK cells in HIV-1 infection, mechanisms behind T cell activation and finally speculating how ART may restore the innate and adaptive systemic immune dysfunction caused by persistent HIV-1 infection.

5.1 Importance of reproducible PBMC processing and analysis in resource limited settings

PBMC isolation and cryopreservation is essential for studies with complex immunological questions. Most current HIV-1 vaccines and natural history studies are critically dependent on the ability to isolate, cryopreserve and thaw PBMC samples of high quality. In **PAPER I**, we characterized the yield, viability, phenotype and function of PBMC from HIV-1 infected and uninfected Ugandans and outlined methods to ensure quality. Use of Leucosep® tubes for PBMC separation reduced granulocytes and platelets, while enriching for lymphocytes and monocytes. B cells exhibited a significant decrease following processing and cryopreservation in both HIV−1 infected and uninfected subjects but all other major lymphocyte subsets were preserved at comparable rates. Cryopreservation did not appear to impact function as specimens exhibited low background and responded to staphylococcal enterotoxin B (SEB) by IFN-γ and IL-2 production. Long-term cryopreservation of samples stored for greater than three years did not impact yield or viability regardless of HIV-1 infection status. We have developed a comprehensive internal quality control (QC) program to monitor PBMC processing in order to guarantee that these cells will be suitable for vaccine trial immunogenicity and natural history studies.

5.1.1 Phenotype of cryopreserved PBMC

PBMC processing and cryopreservation can impact the ability to assess certain lymphocytes. In **PAPER I,** B cell frequencies were decreased in both HIV-1 infected and uninfected volunteers, indicating particular sensitivity of B cells to the PBMC isolation and cryopreservation process (**PAPER I, Fig. 1D and 1E**). Interestingly, we observed no difference in T cell or NK cell distribution, strengthening the results generated in this thesis project. However, more recent data shows that NK cell processing can alter phenotype and function of this lymphocyte subset. Naranbhai and colleagues showed that exposure of NK cells to the rigors of PBMC isolation using ficoll and centrifugation marginally alters the expression of activation markers (CD69 increased and CD38

decreased) and chemokine receptors (CCR4 increased and CCR7 decreased) as compared to whole blood levels in healthy women from South Africa[207]. Furthermore, they showed that delays in processing samples (greater than 8 hours) can result in similar alterations in phenotype and may diminish the ability of NK cells to respond to phorbol-12-myristate-13-acetate and ionomycin, both by degranulation and cytokine production[207]. Consequently, future studies examining activation, function and trafficking of NK cells should consider the effects of PBMC processing. In our studies, PBMCs were processed within 8 hours of blood collection, and we demonstrate that high quality cryopreservation in a high throughput specimen-processing laboratory can be achieved in the resource limited setting. Other groups have also shown that delays in PBMC separation can result in deleterious effects such as activation of granulocyte populations that inhibit T cell function[208]. **PAPER I** outlines methods to monitor contaminating granulocytes and to reduce possible contamination through training of technical staff on strategies to harvest less Ficoll beneath the PBMC layer, where granulocytes are found (see Thesis Fig. 7B). In addition, methods to monitor processing time and other quality indicators are crucial, particularly in settings where clinics or blood collection is conducted off site from the processing center. Unfortunately, we were unable to compare cryopreserved NK cell phenotype or function to freshly isolated specimens and future studies need to assess the impact of processing and cryopreservation on NK cell research.

5.1.2 Function of cryopreserved PBMC

The purpose of PBMC cryopreservation is to store lymphocytes and monocytes in a sufficient manner to allow for the batching and post study analysis of samples, particularly when fresh assays are not feasible. In order to qualify processing procedures, downstream functional and phenotypic assays should be conducted in order to ensure effectiveness of preservation techniques. To that effect, in **PAPER I** we show simple ways to monitor the quality of PBMC separation/cryopreservation both by viability and post thaw yield, but also in functional *in vitro* assays that detect cytokine production by intracellular flow cytometry in response to various stimulation conditions (**PAPER I, Fig. 3A and 3D**). Furthermore, we demonstrate that long-term cryopreservation of PBMC, in both HIV-1 uninfected and HIV-1 infected individuals, does not impact the quality or quantity of PBMC post thaw. Accordingly, the procedures used to process, store and thaw PBMC are adequate for the down-stream immunology assessment that we performed in this thesis. Since the publication of **PAPER I** in *Clinical and Vaccine Immunology*, additional papers have been published regarding optimal processing and storage conditions for T cell based assays. Many processing centers in the resource limited setting lack access to liquid nitrogen and the ability to store PBMC below -70˚C. Weinberg et al. report that longer storage of cells at -70˚C decreased the post-thaw viability and antigen specific ELISpot responses in both HIV-1 infected and uninfected study participants, although qualitatively ELISpot responses did not change[209]. Another study looking at processing and storage factors that impact *in vitro* assays, showed that prolonged storage without agitation prior to PBMC processing can lead to decreased ELISpot responses and higher background in tetramer staining by flow cytometry[210]. Poor PBMC processing technique and prolonged storage most likely leads to increased apoptosis and decreased quality of the PBMC product. A novel study to test the hypothesis that the level of programmed cell death after cryopreservation

could alter downstream functional assays showed that the presence of late apoptotic cells was associated with decreased ELISpot values in response to viral lysates and peptides[211]. There are a few methods to reduce some of the potential negative effects of poorly cryopreserved specimens. For example, suboptimal cryopreservation can lead to clumping of cells post thaw, a process thought to result from excessive cell death and release of DNA. The use of DNAse minimizes clumping and does not affect lymphocyte assessment[212].

5.2 NK cells in HIV-1 infection

NK cells are innate lymphocytes that play a significant role in the control of viral infections, including HIV-1. In **PAPER II** we examined the state of the NK cell compartment in Ugandans with untreated HIV-1 infection in comparison with matched uninfected controls and investigated possible associations between NK cells and markers of disease progression. The function and phenotype of NK cells were investigated using 9-color polychromatic flow cytometry analysis of cryopreserved PBMC. Interestingly, low CD4 counts were associated with increased levels of IFN-γ and degranulation in CD56bright NK cells. Also noteworthy, the results of this investigation of indicated that HIV-1 infected Ugandans display elevated NK cell activity, despite the altered functional and phenotypic NK cell profile.

5.2.1 NK cell normal distribution in Ugandans

NK cell absolute counts and percentages, defined as CD45+, CD3-, CD16 or CD56+, CD19- cells, are a standard part of the immunophenotyping panel used to assess the lymphocyte distribution in cohorts we work with. Clinical phenotyping panels are processed using a fresh whole blood, lyse no wash procedure. In developing flow cytometry reference intervals for Ugandans, we analyzed 654 normal healthy adults (**unpublished findings**) and found that NK cells constitute 14% (5 – 32% reference interval of 95%) of lymphocytes in whole blood while absolute counts of NK cells varied substantially (86 - 854 cells/μl). Gender differences were observed with women exhibiting significantly lower NK cell levels than men in both percentage and absolute counts (12% vs 15% and 283 vs 341 cells/μl, respectively)(both $P<0.001$). As it has been previously reported that NK cell frequency is elevated in Asians as compared to Caucasians[213-216], we were interested to compare any potential differences between this Ugandan population and other more characterized cohorts in the US and Europe where NK cells make up a mean 13% of lymphocytes (95% range = 5 – 26%) and absolute counts ranged from 84 - 724 cells/μl. No statistically significant difference was observed between these geographically disparate locations. We also found that NK cell frequency was similar to normal values reported in Kenya[217] and Tanzania[218].

5.2.2 $CD56^{neg}CD16^{+}$ NK cells in chronically HIV-1 infected Ugandans

In **PAPER II** we identified trends in HIV-1 infection where CD4+ T cells are declining concomitant to increasing viral load. However, we observed that the overall absolute counts of NK cells remain unchanged (**see PAPER II, Fig. 1A**). Despite the appearance of consistency within the NK cell compartment, when looking at NK cell subsets we observed differences suggesting alterations due to HIV-1 infection. As mentioned in the introduction, NK cells can be divided into subsets based on the expression of CD56 and

CD16 where the $CD56^{bright}CD16^{-}$ phenotype is associated with cytokine and chemokine production, the $CD56^{dim}CD16^{+/-}$ phenotype is associated with cytotoxicity, and the $CD56^{neg}CD16^{+}$ profile marks an aberrant NK phenotype that is developed in HIV-1 infection[93,96,107,219]. Contrary to previous reports, our data would suggest that $CD56^{neg}CD16^{+}$ NK cells are not anergic, but may display an altered functional profile. We see that MHC^{null} K562 cell-stimulated PBMC in HIV-1 infected study participants responded with increased degranulation and MIP-1β production as compared to HIV-1 uninfected individuals **(see PAPER II, Fig. 3F)**. Moreover, unstimulated $CD56^{neg}CD16^{+}$ NK cells exhibited an activated state with increased cytokine (IFN-γ), chemokine (MIP-1β) and degranulation (CD107a) expression **(see PAPER II, Fig. 3F)**. In fact, NK cells generally displayed elevated production of IFN-γ, MIP-1β, as well as CD107a in HIV-1 infected subjects (see **PAPER II, Fig. 3**). The ontogeny of $CD56^{neg}CD16^{+}$ NK cells is still unclear, although there has been some recent focus on what is driving this phenotype in chronic infections such as HIV-1. One current model suggest that $CD56^{neg}CD16^{+}$ NK cells differentiate from $CD56^{dim}CD16^{+/-}$ NK cells, and may represent a distinct functional subset of NK cells with preferential function to produce antiviral chemokines[96]. The role of persistent viremia on the size of this subset is unclear, although it appears that higher viral loads are associated with higher frequencies of $CD56^{neg}CD16^{+}$ NK cells thereby suggesting that viral burden leads to the altered phenotype and function of this NK cell subset[220,221]. It is interesting that $CD56^{neg}CD16^{+}$ NK cells express low levels of CCR7, HLA-DR, NKG2A, intermediate levels of CD57, while most of these cells express the activation marker CD38 and the β chain of the IL-2/IL-15 receptor, CD122[222]. *In vitro* assays exploring differences in CD56- and CD56+ NK cells from HIV-1 infected participants show that stimulation with recombinant human IL-2 completely restores CD56 levels after 21 days in culture with simultaneous increases in proliferation[219]. IL-2 treatment had little effect on activating and inhibitory receptor expression and was insufficient to restore function, as measured by K562 killing[219]. In many ways, the expansion of $CD56^{neg}CD16^{+}$ NK cells resembles the build-up of terminally differentiated exhausted CD8+ T cells observed in the highly activated environment of chronic HIV-1 infection, but this model does not completely fit[96]. Therefore, more information is needed to determine if $CD56^{neg}CD16^{+}$ NK cells are exhausted due to persistent HIV-1 antigen exposure, or if this aberrant phenotype results from a distorted maturational process.

5.2.3 NK cell control of HIV-1 infected CD4+ T cells

A model where high HIV-1 viral load in chronic infection directly drives expansion and dysfunction of NK cells remains to be proven. In fact, the direct ability of NK cells to recognize and kill autologous CD4+ T cells has proven difficult to replicate *in vitro*[223,224]. Inhibitory KIR molecules recognize changes in the level of MHC class I molecules on the surface of cells. Matthew Bonaparte and colleagues showed that despite a major reduction in surface expression of class I molecules on HIV-1 infected cells, NK cells were not able to lyse autologous *in vitro* infected CD4+ T cells[223]. The same authors later reported that in uninfected donors, *in vitro* HIV-1 infection of activated and proliferating autologous CD4+ T cells reduced expression of HLA-A and HLA–B molecules, but did not alter the level of HLA-C or HLA-E[224]. In addition, blocking of the HLA-C and HLA-E interaction released the inhibitory signal and increased killing was

observed, suggesting that HIV-1 alterations and down-regulation of class I molecules may make these cells more susceptible to NK cell killing[224]. In addition to loss of inhibitory signals, activating signals are necessary to stimulate NK cell killing. Jeffrey Ward et al. showed that ligands for NKp30 or NKp46 are not present on infected CD4+ T cells. However, several ligands for NKG2D are induced including ULBP-1, ULBP-2, and ULBP-3[225]. One potential mechanism of NKG2D ligand up-regulation is through the action of the HIV-1 protein Vpr[226]. Moreover, Manuela and colleagues showed that HIV-1 infected donor-derived NK cells are only able to kill autologous endogenously infected CD4+ T cells through NKG2D mechanisms, while reduced NCR ligands and expansion of $CD56^{neg}CD16^{+}$ NK cells leaves many NK cells unable to respond effectively to infected CD4+ T cells[227]. Another argument would suggest that NK cell dysfunction is a consequence of HIV-1 burden, but may not be associated with direct control of viral load, evidenced by comparing immunologic controllers, ART-suppressed individuals and non-controllers[221]. Still, other mechanisms exist whereby NK cells can contribute to control and elimination of CD4+ T cells. As discussed in **PAPER II**, up-regulation of NKp44 ligands is associated with co-receptor usage and inversely proportional to CD4+ T cell absolute counts in infected monkeys[228]. Additionally, NKp44L expression is induced on bystander CD4+ T cells in the presence of soluble gp41[229]. Our data in **PAPER II** supports a model where NKp44 could be associated with CD4 elimination (see **PAPER II, Fig. 4A**). Irrespective of the numerous reports of how NK cells can potentially limit viral load and or kill CD4+ T cells, more information is needed to better understand the mechanisms that are associated with control of virus. This may not be possible to determine through studies of chronically HIV-1 infected individuals, as the immune pressure exerted by NK cells may have been escaped by the virus at this period in disease progression.

5.2.4 KIR genotype, NK cell KIR phenotype and HLA-B Bw4 80I

Genetic association studies have implicated NK cells as major contributors to control of HIV-1. KIR receptors and their HLA class I ligands have been intensely studied, particularly in HIV-1, because *KIR* and *HLA* genes are highly polymorphic and certain KIR-HLA interactions could influence differences between individuals in HIV-1 disease progression[98,100]. The two *KIR* genes *KIR3DL1* and *KIR3DS1*, which are alleles of the same locus, and the inhibitory and activating receptors they encode, are both associated with slower HIV-1 disease progression when found in combination with their HLA ligand[230-233]. In **PAPER II**, we show a reduction in KIR2DL1 expression, unchanged KIR2DL2/DL3 expression and an increase in KIR3DL1 expression in certain NK cell subsets (see **PAPER II, Fig. 2C - 2F**). To better understand the role of KIR receptors in this cohort from Kayunga district, we analyzed KIR and the corresponding HLA-ligands at the genetic level. Sequence-specific priming (SSP) real-time PCR was used to genotype for *KIR3DL1*/*KIR3DS1*, *KIR2DL2*/*KIR2DL3* and their HLA-class I ligands as previously described[234]. *HLA-B Bw4* or *Bw6* was determined, allowing the discrimination of *Bw4* alleles having isoleucine or threonine at position 80 corresponding to KIR3DL1 ligands. Similarly, *HLA-C group C1* or *C2* was determined because these are KIR2 ligands. The results are presented in **PAPER IV**. The presence of *HLA-B Bw4-80I* was associated with elevated frequencies of KIR3DL1+ NK cells in chronically HIV-1 infected Ugandans. Furthermore, a positive correlation was observed between the size of the

KIR3DL1-expressing NK cell subset and viral load, and, importantly, this pattern was observed only in *Bw4-80I*+ patients.

Preferential expansion of KIR3DL1+ NK cells in the presence of *HLA-B Bw4-80I* may at first glance seem to support a model whereby this genetic combination provides some level of virologic control. However, our data may not necessarily support a beneficial relationship between expansion of the KIR3DL1+ NK cells in the presence of certain *HLA-B* alleles. In fact, we see a positive correlation between HIV-1 viral load and the KIR3DL1+ NK cell frequency in the presence of *HLA-B Bw4*, which may indicate that this phenotype is associated with increased viral replication in chronic HIV-1 infection (**PAPER IV, Fig. 2A**). HIV-1 is known to down-regulate the expression of MHC class I molecules (HLA-A and HLA-B) on the surface of infected CD4+ T cells[235,236], which leaves these cells as potential NK cell targets according to the missing self hypothesis. As mentioned above, an additional activating signal is needed to stimulate these NK cells such as NKG2D ligands induced by, for example, HIV-1 Vpr[226]. More recently, Vpu-mediated down-regulation of the receptor NK, T and B cell antigen (NTB-A) has been associated with an incomplete activation signal that results in reduced NK cell degranulation and cytotoxic ability[237]. It is tempting to speculate that this incomplete stimulation could result in expansion and proliferation of the KIR3DL1+ NK cell subset, while not providing sufficient stimulation necessary for killing of the infected CD4+ T cell target. This is highly speculative and would need to be tested. This model may be supported by data in mice where murine CMV (MCMV) infection was associated with a biphasic expansion of NK cells [122,238]. The initial phase was associated with proliferation and production of IFN-γ independent of the activating KIR-equivalent in mice, Ly49H, while the second phase was a specific Ly49H-dependent expansion[122,238]. Another possible explanation for the expansion of KIR3DL1+ NK cells may involve the peptides that bind the HLA-B Bw4 groove. Lena Fadda and colleagues show that an altered repertoire of peptides in HLA-C can disrupt the inhibition provided normally through KIR2DL2 and KIR2DL3[239]. The same could be true for certain HIV-1 peptides binding to HLA-B alleles with Bw4 motifs, thereby reducing KIR3DL1 inhibition. Irrespective of the possible mechanisms of expansion, there still exists the issue of viral control demonstrated by numerous genome-wide association studies where KIR3DL1 and HLA-B Bw4 are linked to lower viral load and slower disease progression[98,230-233]. It is important to note that our study is limited in that we are looking cross-sectionally in chronic untreated infection. Galit Alter et al. showed a preferential expansion of KIR3DL1+ NK cells and increased KIR3DL1 mRNA in individuals with *Bw4 80I* in acute infection. As we discuss in **PAPER IV**, it is possible that the major protective effect of KIR3DL1 may be exerted early in HIV-1 infection.

5.2.5 Increased CD56^{dim}NK cell polyfunctionality in HIV-1 infection

In addition to the increased frequency of KIR3DL1+ NK cells, we observe that NK cells are more polyfunctional with regard to CD107a, IFN-γ, and MIP-1β in HIV-1 infected patients as compared to uninfected people (**PAPER IV, Fig. 3**). We go on to show that the KIR3DL1+ NK cells in *Bw4+* individuals are particularly responsive to K562 cells by production of increased IFN-γ and MIP-1β (**PAPER IV, Fig. 4**). This data is consistent with a previous report showing that KIR3DL1 in the presence of the cognate HLA class I

ligand license NK cells to have increased function[240]. Together, these two papers indicate that KIR3DL1+ NK cells in *Bw4+* hosts are able to produce more anti-viral cytokine (IFN-γ), pro-inflammatory cytokine (TNF-α), and CC-chemokine (MIP-1β) that may limit HIV-1 infectivity. Another mechanism that NK cells can muster to participate in viral control is the direct lysis of HIV-1 infected CD4+ targets. When stimulated with K562 cells, thereby releasing KIR3DL1 inhibition, we observe increased ability of NK cells to degranulate (**PAPER IV, Fig. 3**). One model in HIV-1 infection is that KIR3DL1 inhibition is released by HIV-1 infected CD4+ T cells due to down regulation of HLA-A and HLA-B molecules, leaving the target cell susceptible to lysis[241]. But then why do we not see associations with virologic control? The measure used to assess NK cell responsiveness are MHC^{null} K562 cells, which may not accurately represent the HIV-1 infected CD4+ T cell targets *in vivo*. Again referring back to the work by Shah et al., decreases in the amount of NTB-A may result in inadequate signaling needed for killing of target cells[237]. Furthermore, insufficient "co-stimulation" that can reduce the cytotoxic potential of NK cells and reduce their capacity to produce IFN-γ and TNF-α[242]. Another potential benefit from the presence of HLA-B Bw4 is that KIR3DL1+ NK cells display less activity in unstimulated conditions **(data not shown)**. All three functional markers we assess in our overnight assay display elevated basal levels in HIV-1 infected patients homozygous for HLA-B Bw6, particularly in the $CD56^{dim}CD16^{+/-}$ and $CD56^{neg}CD16^{+}$ NK cell subsets. The fact that the patients with at least one HLA-B Bw4 allele exhibit lower unstimulated IFN-γ, MIP-1β and degranulation may indicate that these cells will contribute less to an inflammatory environment leading to less generalized immune activation, which in turn may slow disease progression. Ultimately, more information is needed to better understand how NK cell function, particularly in certain HLA and KIR combinations, can contribute to control of virus replication and HIV disease.

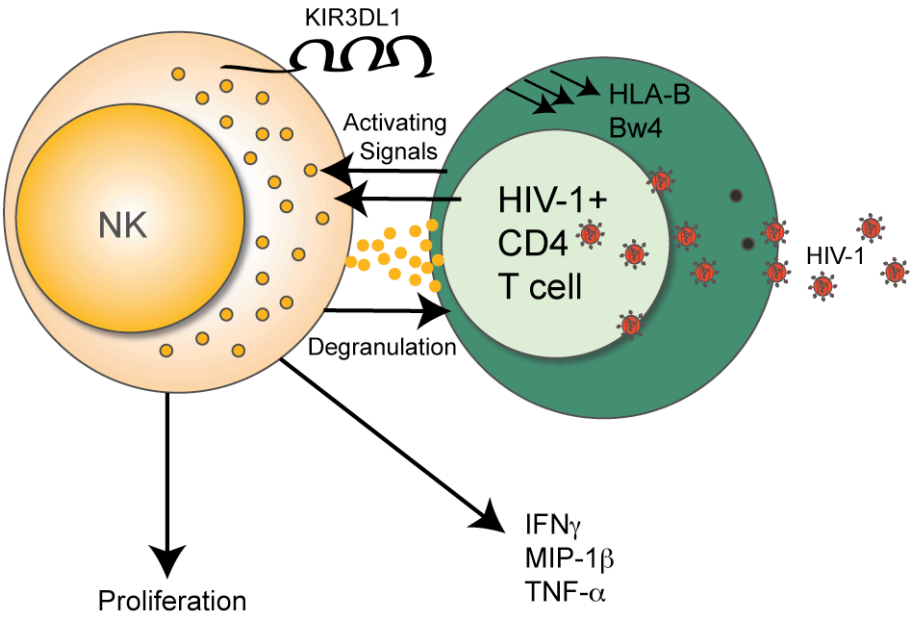

Figure 10. Potential mechanisms of NK cell mediated control of HIV-1 viremia in HLA-B Bw4 individuals.

5.2.6 NK cell memory

It is tempting to ask if the expansion of KIR3DL1+ NK cells may represent a sort of NK cell memory in humans, similar to what has been reported in the MCMV model. The activating Ly49H receptor recognizes the viral protein m157, a MHC class I-like decoy molecule, and has been implicated in "antigen-specific" NK cell-mediated response to CMV infection[243,244]. In this model of NK cell memory, initial recognition of infected DCs through Ly49H is accompanied by inflammatory cytokines such as IL-12, which in turn induce NK cells to secrete cytokines, mediate cytotoxicity, and proliferate to expand an effector pool ultimately seeding the memory population[125]. It is, however, difficult to relate the function of the activating Ly49H receptor in MCMV infection to the inhibitory receptor KIR3DL1 in HLA-B Bw4+ HIV-1 infected humans. In fact, murine inhibitory Ly49C/I+ NK cells are less protective than Ly49C/I- cells, both by adoptive transfer and depletion studies, suggesting that inhibitory receptors may not be beneficial in this model[245]. Cooper et al. suggest a model of NK memory where NK cells are non-specifically activated by inflammatory cytokines and these NK cells in addition to producing cytokines and mediating pathogen control, can seed a population of memory NK cells with higher functional potential upon restimulation[246]. This model may not sufficiently explain the expansion of KIR3DL1+ cells either. More information is needed to characterize the phenotype and function of memory NK cells to better define these subsets in chronic disease. As mentioned earlier, memory hepatic NK cells sensitized to HIV antigen express CXCR6, but this was determined in a murine model where mice were administered virus-like particles expressing HIV-1 antigen to measure memory[126]. This needs to be explored in humans, but may prove challenging based on the compartment where these memory cells are normally distributed.

5.2.7 NK cell relationship to HIV-1 disease progression in Ugandans

Numerous alterations are observed to the NK cell compartment in Ugandans with chronic HIV-1 infection. These changes seem to be independent of viral subtype. as determined by comparison of HIV-1 subtype A and D, which represent the most common strains found in Uganda. We see an altered distribution of NK cell subsets with an accumulation of $CD56^{neg}CD16^{+}$ NK cells and decreased $CD56^{dim}CD16^{+/-}$ NK cells. Surface phenotype is changed with decreases observed for the inhibitory receptors CD161, NKG2A, KIR2DL1 and decrease in the activating receptor NKp30 (**see PAPER II, Fig.2**). These quantitative and qualitative changes in the NK cell compartment may be due both to viral antigen exposure and to the overall immune status in HIV-1 chronic infection. The phenotypic and functional changes found in **PAPER II** were not associated with viral load, and occur in a context where overall NK cell frequency is directly proportional to CD4+ T cell counts (**see PAPER II, Fig.1**). Furthermore, several parameters were found to be inversely proportional to CD4+ T cell counts, particularly in the $CD56^{bright}CD16^{-}$ NK compartment. This compartment is generally considered to be less mature or differentiated. In our studies, we observe that HIV-1 infection is associated with significantly higher $CD56^{bright}CD16^{-}$ NK activity in response to K562 cells with increased IFN-γ and CD107a (see **PAPER II, Fig. 3**). This heightened functional capacity in $CD56^{bright}CD16^{-}$ NK cells is inversely proportional to absolute CD4 counts, suggesting a link to the decay of the immune system. Additionally, the $CD56^{bright}CD16^{-}$ NK cell subset is immuno-modulatory, suggesting a more supportive

role in the adaptive immune response through production of cytokines. $CD56^{bright}CD16^{-}$ NK cells are found at higher frequency in secondary lymphoid tissue, areas rich in other immune cells that help direct adaptive immune responses[120]. It is interesting to note that $CD56^{bright}CD16^{-}$ NK cells are particularly adept at responding rapidly to innate signals. Macrophages stimulated with LPS induce $CD56^{bright}CD16^{-}$ NK cells to produce 6-fold higher amounts of IFN-γ compared to $CD56^{dim}CD16^{+/-}$ NK cells[247]. LPS and other microbial products can cross the compromised, CD4+ T cell-depleted gut barrier. This contributes to increased levels of immune activation, a hallmark of chronic HIV-1 disease progression[248]. Furthermore, NK cells can exert anti-HIV-1 functions (including IFN-γ) in a CD4+ T cell-dependent manner[249]. NK cells modulate DC function and maturation in a contact-dependent manner contingent upon TNF-α production. Moreover, DCs and NK cells provide direct feedback in a reciprocal manner, enhancing function and maturation of both cell types[250]. Interestingly, we observe reduced levels of NKp30 expression in HIV-1 infected compared to uninfected individuals (**PAPER II, Fig. 2**), and this activating receptor has been shown to be important in NK cell recognition of DC[251]. The cytokine environment also tightly regulates NK cell function[252]. Indeed, this data may suggest that a delicate balance exists between multiple arms of the immune system, and the alterations we observe in the NK cell compartment may be the combination of an imprint of chronic infection and a directed response to HIV-1 viral load.

5.3 T cell activation in chronic HIV-1 infection

HIV-1 infection results in a state of persistent immune activation that involves T-cells and is characterized by increased surface expression of CD25, CD38, CD69, HLA-DR, β2microglobulin and neopterin[174]. The most common measures of activation in T cells are CD38, an ectoenzyme important to the metabolism of T lymphocytes, and HLA-DR. The degree of immune activation for any HIV-1 infected individual is widely variable, but during chronic infection it is associated with level of viremia, CD4+ T cell counts, and disease progression[253-256]. In addition, striking correlations between disease progression and immune activation suggested prognostic value of monitoring activation markers for patients on antiretroviral therapy[257,258]. It is thought that baseline activation is higher in HIV-1 negative Africans due to endemic infections and poorer living conditions, as compared to HIV-1 uninfected people in Europe and North America[259]. Additionally, dampening of the immune response due to cellular exhaustion may play a role in loss of control of HIV-1 viremia. PD-1 (CD279), is a negative regulator of TCR signaling and T cell activation[161]. PD-1 expression on HIV-specific CD8+ T cells is associated with poor function of these cells and disease progression [260-262]. In **PAPER III**, we examined expression of CD38, HLA-DR and PD-1 on T cells in chronically infected Ugandans from the rural district of Rakai. As expected, all three markers were elevated on CD8+ T cells when comparing HIV-1 infected individuals to uninfected community matched controls, both by frequency and mean fluorescence intensity (**data not shown**). Similar increases were observed in CD4+ T cells. What became most notable, however, was the co-expression of these three markers, particularly on CD4+ T cells, where HIV-1 infection was associated with profound increases compared to uninfected people (**PAPER III, Fig. 1B**). These cells were CD45RO+, CCR7-, CD28+, with

an increased frequency of Ki67, indicating these cells were effector memory cells undergoing proliferation (**PAPER III, Fig. 1C**).

5.3.1 CD4+ Deregulated Effector Memory T cells

T_{regs} down-regulate the immune response in both CD4+ T cells and CD8+ T cells[263]. Loss of Tregs due to HIV-1 infection may be associated with the hyperimmune activation and subsequent CD4 depletion observed in natural infection [264]. Some of the markers commonly used to identify $T_{regs,}$ including CD25, CD127, and FOXP3, were used to quantify these cells in the Rakai cohort. We observed a statistically significant decrease in frequency of T_{regs} in HIV-1 infected subjects compared to HIV-1 negative individuals (**PAPER III, Fig. 2A**). The frequency of T_{regs} was not associated with viral load or CD4+ T cell counts, but was inversely proportional to the level of CD38, HLA-DR and PD-1 triple-positive CD4+ T cells (**PAPER III, Fig. 2B-D**). This indicated that the phenotype of these activated CD4+ T cells represented a deregulated effector memory subset (CD4+ T_{DEM}). Interestingly, the frequency of CD4+ T_{DEM} was correlated directly to viral load and inversely to CD4+ T cell absolute counts (**PAPER III, Fig. 3A-B**). In addition, individuals who had higher levels of CD4+ T_{DEM} cells (defined as frequency above the median) progressed faster to AIDS compared to those with levels below the median (**PAPER III, Fig. 3C**). The initial positive correlation of CD4+ T_{DEM} cells with HIV-1 viral load suggests that this activated phenotype is driven by viral antigen, or alternatively that increased immune activation may fuel viral replication. Moreover, a combination of events that challenge the immune system could actually contribute to the systemic immune activation and the appearance of CD4+ T_{DEM} cells that are highly associated with disease progression.

5.3.2 CD4+ T_{DEM} cells and microbial translocation

One primary driver of immune activation in HIV-1 infection is thought to be microbial translocation across a compromised "leaky" gut[265]. The premise of this suspected mechanism is that mucosal CD4+ T cells are preferentially depleted early in acute HIV-1 infection and are never recovered, allowing for increased permeability across the gut membrane by certain microbial components like LPS[266,267]. Original observations suggested that plasma levels of LPS are directly correlated with T cell activation in HIV-1 infected patients[266], even in HIV-1 elite controllers where a more gradual depletion of CD4+ T cells is observed[268]. To support the role of microbial product translocation, the natural simian immunodeficiency virus (SIV) infection in African green monkeys, mandrils, and sooty mangabeys provide insight into disease progression, where in the presence of high viremia and absence of cellular activation and peripheral markers of microbial translocation, monkeys do not progress to a diseased condition[269,270]. What is more, African green monkeys that are given LPS display increased levels of immune activation and exhibit higher levels of viral replication, possibly due to increased levels of CD4+ T cell targets[271]. Other markers of microbial translocation include soluble CD14 (sCD14), endotoxin-core antibodies (EndoCAb), and bacterial ribosomal DNA 16S (16S rDNA), all of which associate with T cell activation in chronic HIV-1 infection[248,266]. Monocytes, when activated with microbial products such as LPS, will release sCD14 from the surface of the cell[266,272]. In **PAPER III** we observe elevated levels of sCD14 that are directly proportional to CD4+ T_{DEM} cells (**PAPER III, Fig. 5A**), directly proportional to viral load (**PAPER III, Fig. 4C**), and inversely proportional to CD4+ T cell absolute

counts (**PAPER III, Fig. 4B**). Together this data supported a model of microbial translocation. However, we did not observe a difference in the rates of disease progression between individuals with high levels of sCD14 compared to individuals with low levels of sCD14 (**PAPER III, Fig. 4D**), suggesting that maybe sCD14 is linked to immune activation but less associated with disease progression. It is important to note that our data is in contrast to a recent report where plasma levels of sCD14 were independently predictive of disease progression and mortality. In this report, although the difference in levels of sCD14 between groups was statistically significant, it did not appear to be biologically significant[273]. Interestingly though in this study, LPS was not associated with disease progression, indicating that activation of monocytes and shedding of sCD14 may not be dependent on LPS[273]. In addition, the investigators compared the upper quartile and lower quartile of their cohort, while we compared levels above and below the median. Our study is in line with another longitudinal study, in Uganda, where sCD14 levels demonstrated little relationship with disease progression[274]. Interestingly, in the *Macaca mulatta* model, microbial translocation was determined not to be involved in disease progression, as levels of sCD14 and LPS were not different between animals who progressed fast or slow[275]. More studies are needed to examine the role of sCD14 in immune activation as this molecule may represent an independent marker of monocyte activation that is associated with T cell activation.

In addition to the sCD14 being cleaved from the surface of monocytes, LPS induces the production of IL-6. Moreover, HIV-1 infection is associated with increased plasma IL-6 levels[276,277]. We observed elevated levels of IL-6 in HIV-1 infected individuals that are directly proportional to CD4+ T_{DEM} cells (**PAPER III, Fig. 5B**), weakly proportional to viral load (**PAPER III, Fig. 4G**), and inversely proportional to CD4+ T cell absolute counts (**PAPER III, Fig. 4F**). Unlike sCD14, we observed faster rates of disease progression in individuals with high levels of IL-6 compared to individuals with low levels of IL-6 (**PAPER III, Fig. 4H**), indicating that IL-6 may be linked to disease progression more so than sCD14. In addition to LPS, the HIV-1 protein Vpr has been shown to induce monocyte production of IL-6[278], indicating a potential direct link between HIV-1 viral antigen and monocyte activation independent of microbial translocation. Furthermore, monocytes from HIV-1 infected individuals were ineffective at responding to LPS, arguing against the contribution of microbial translocation products in driving immune activation[279]. IL-6 has been shown to influence the survival and proliferation of antigen specific memory CD4+ T cells thereby increasing the effector memory pool (reviewed in [280]) and increasing the availability of targets for HIV-1 infection. In experiments supporting **PAPER III**, we were unable to induce CD4+ T_{DEM} cells with soluble IL-6 (**data not shown**). We also did not see any production of IL-6 in the T cell compartment after stimulation with a diverse range of antigens (**data not shown**). However, the long-term impact of IL-6 exposure on the T cell compartment cannot easily be addressed *in vitro*. Taken together, our data may suggest that the impact of chronic HIV-1 infection is parallel in the monocyte and T cell compartments but are not directly linked. The question remains, if microbial translocation is a cause or consequence of HIV-1 disease progression[281].

5.3.3 CD4+ T_{DEM} cells driven by diverse antigens, innate and bystander activation

In an attempt to identify the cause of the aberrant immune activation and accumulation of CD4+ T_{DEM} cells, we stimulated cryopreserved PBMC with an array of antigens *in vivo* to examine the development of these cells. We observed that Candida, CMV, and other recall antigens were able to induce this phenotype after 3-6 day stimulation periods (**PAPER III, Fig. 6A**). We also observed that the CD4+ T_{DEM} cells were enriched for virus (CMV and HIV-1) specific cells using an intracellular assay to measure IFN-γ and TNF-α production (**PAPER III, Fig. 6C**). We were, however, surprised when we observed no difference in TCR Vβ distribution in the CD4+ T_{DEM} cells as compared to the overall CD4+ T cell compartment (**PAPER III, Fig. 6D**). This data would suggest that a diverse array of microbial antigens inclusive of, but not limited to, products translocating across the gut membrane can induce CD4+ T_{DEM} cells, but the relative contribution to HIV-1 disease progression still remains unclear. Other mechanisms of immune activation include innate production of IFN-α and bystander activation. pDC are innate cells that are able to produce interferons in response to viruses (reviewed in [78]). It is proposed that pDC are able to recognize HIV-1 through TLR7 and respond with vigorous IFN-α production that may mediate immune activation[282,283]. In fact, Angela Meier showed increased frequency of CD38+CD8+ T cells in direct response to IFN-α, after 20 hours *in vitro* culture[284]. In the murine model, repeated stimulation of TLR7 using receptor agonist R848 lead to lymphopenia, increased inflammatory cytokines and altered lymphoid architecture, which may resemble HIV-1 immune activation conditions[285]. As mentioned in **PAPER III**, we tried to induce CD4+ T_{DEM} cells through stimulation with IFN-α, however we were unable to generate the phenotype associated with HIV-1 disease progression (**data not shown**). We did not observe upregulation or increased frequencies of CD38+ T cells indicating that we were unable to replicate the conditions where IFN-α may contribute to development of CD4+ T_{DEM} cells.

Bystander activation is the non-specific activation and expansion of T cells in response to an infection or inflammatory condition. Early investigations into the extent of bystander activation examined groups of mice challenged with a number of viral pathogens in order to assess the extent of heterologous T cell activation[286-290]. Stephan Ehl showed that bystander activation in mice both *in vivo* and *in vitro* was dependent on cytokines such as IL-2 but not type I interferons like IFN-α[286]. Interestingly common gamma chain cytokines like IL-2 have been associated with increases in PD-1 expression[291], as is observed on our CD4+ T_{DEM} cells. Another group showed that murine bystander activation was dependent on IFN-γ[290]. In non-human primates, absence of bystander activation was associated with nonpathogenic SIV infection in sooty mangabeys[292]. In humans during primary HIV-1 infection, CD8+ T cells specific for multiple viruses including Epstein-Barr virus, CMV, and influenza virus up-regulate CD38 directly proportional to HIV-1 viral load[293]. Furthermore, bystander activation was associated with increased levels of Ki67 in primary HIV-1 infection[293]. Concordantly, we observe an expansion of HIV-1 and CMV specific CD4+ T_{DEM} cells. However, our data is from chronic infection and CMV specific cells outnumber HIV-1 specific cells. In addition, we see increased Ki67 expression in CD4+ T_{DEM} cells thereby supporting a model of bystander activation. Taken together our data from **PAPER III** supports several proposed models of pathogenic immune activation, but unfortunately

do not define one precise mechanism that is responsible for the generation of CD4+ T_{DEM} cells. It is likely that the different proposed mechanisms are not mutually exclusive and are somehow tied together both directly and indirectly leading eventually to immunodeficiency and disease progression.

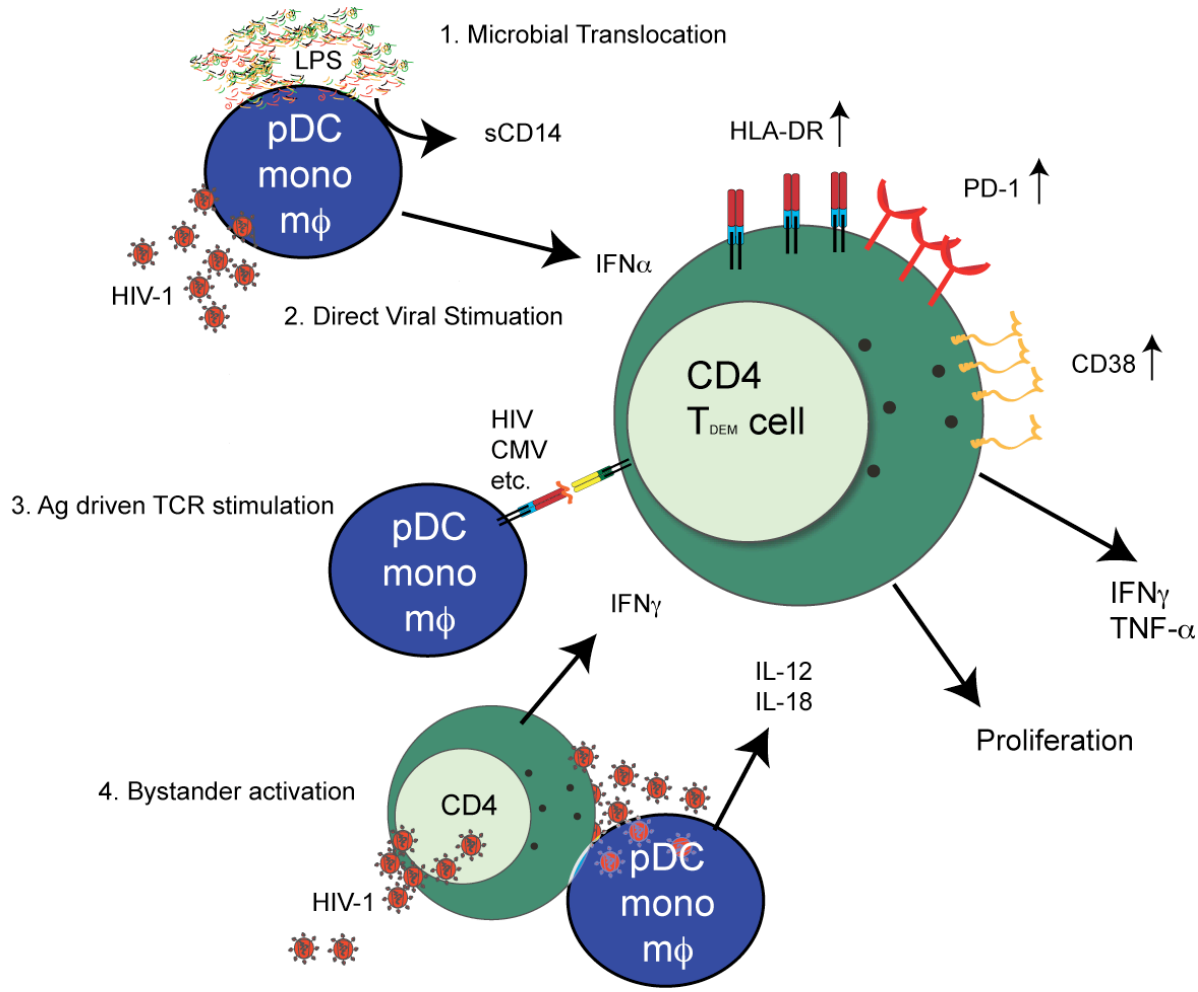

Figure 11. Hypothetical mechanisms behind development of CD4+ T_{DEM} cells in chronic HIV-1 infection.

5.3.4 Will CD4+ T_{DEM} cells restore after initiation of ART?

Studies of immune activation in HIV-1 infected patients, initiating ART, have shown that levels of CD38 and HLA-DR are reduced on CD8+ T cells in parallel to viral load decline[257,258]. Several studies have shown that despite the positive relationship between viral load and CD8+ T cell activation, CD4+ T cell recovery on ART is not predicted by residual levels of T cell activation[294-296]. We have identified pathogenic activation in the CD4+ T cell compartment that is predictive of disease progression, but it is unknown if the size of this subset will contract in response to ART. Consideration of the hypothetical causes behind the expansion of CD4+ T_{DEM} cells may help predict the effect

of ART on these cells. Wei Jiang *et al.* showed that bacterial 16S rDNA was associated with higher levels of CD8+ T cell activation and lower levels of CD4+ T cell restoration in response to ART[248]. This data suggests that irrevocable damage to the GALT during HIV-1 infection can limit the success of ART, and microbial translocation may persist along with CD4+ T_{DEM} cells despite virologic suppression. This is not the same for all mucosal sites as the lower respiratory tract is somewhat spared from the massive depletion that is observed in the GALT, and complete CD4+ T cell restoration occurs through proliferation of the resident CD4+ T cell pools[297]. Microbial translocation was shown to persist in a South African cohort with ART-controlled viremia, where reductions in monocyte activation appeared to be linked to virus[272]. It is noteworthy that IL-6 levels were found to be primarily related to opportunistic infections, and sCD14 was primarily linked to LPS levels, independently of HIV-1 viral load indicating that ART may not reduce certain aspects of immune activation[272]. Malaria is a common infection in East Africa that is treated with chloroquine. Shannon Murray and colleagues examined the affects of chloroquine treatment on viral load and immune activation[298]. They showed that despite unaltered viral loads, chloroquine reduced the frequency of CD38+HLA-DR+ CD8+ T cells and proliferation in both CD4+ T cells and CD8+ T cells. Chloroquine treatments are short and the modest levels of reduction in immune activation may be too transient to have therapeutic effect. Anuradha Ganesan tested atorvastatin, a drug used to lower cholesterol and has known anti-inflammatory properties, in HIV-1 infected ART naïve individuals[299]. Interestingly, they did not observe any change in HIV-1 viral loads, but did see some modest changes in activation markers (HLA-DR and CD38) on both CD4+ T cells and CD8+ T cells[299]. Yet another study showed that the central memory and naïve T cell populations were highly predictive of immunologic response to ART, and that the central memory CD4+ T cell populations were inversely proportional to CD8+ T cell activation[300]. The data presented in this study is consistent with a model where cytokines such as IL-7 support T cell homeostasis and could be involved in CD4+ T cell recovery. In fact, the IL-7 receptor, CD127 was shown to be a major determinant of ART CD4+ T cell reconstitution and was negatively associated with CD8+ T cell activation[301]. Moreover, CD4+ T cells, and not CD8+ T cells have been shown to be more responsive to IL-7 and IFN-α in chronic HIV-1 infected patients, and this may in turn drive increased activation, proliferation and CD4+ T cell depletion[302]. Our data indicate that HIV-1 viral antigen is also a driver of immune activation.. Some HIV-1 infected individuals are not able to reconstitute the CD4+ T cell compartment despite viral suppression on ART[303]. In a recent study, standard treatment with the addition of integrase inhibitor raltegravir, had no impact on immune activation in PBMC or GALT, in individuals with incomplete CD4+ T cell restoration[304]. In addition, levels of immune activation are predictive of second line therapy success in HIV-1 patients who are failing first line treatment indicating that activation[305]. Taken together, all this data indicates a complex interaction between the virus and the pathological immune activation that is associated with HIV-1 infection. Investigations of rates of restoration of CD4+ T cells, and the possible reduction of CD4+ T_{DEM} cells, on ART may provide crucial insight into the specific mechanisms that are responsible for disease progression and treatment failure.

6. CONCLUDING REMARKS

More knowledge about the mechanisms of HIV-1 pathogenesis and immune correlates of protection are needed. The HIV-1 epidemic is of particular concern throughout sub-Saharan Africa, where the majority of infections are still found. We aimed to characterize immune cell functions in Ugandans with untreated chronic HIV-1 infection compared to healthy individuals. We examined processing parameters to ensure the highest quality PBMC in order to assess immune responses. We next investigated NK cells in chronic untreated infection in order to identify the phenotype and function in healthy Ugandans and examined alterations to NK cells attributed to HIV-1. We characterized a number of changes to surface receptors and increased frequency of $CD56^{neg}CD16^{+}$ NK cells. Functions were also altered. Through the conduct of NK cell interrogation, increased predominance of KIR3DL1+ NK cells were noted in HIV-1 infected donors who expressed HLA-B Bw4 80I. Furthermore, we saw increased functional capacity of KIR3DL1+ NK cells in these individuals. Finally, we explored the role of T cell activation in chronic HIV-1 infection. We observed accumulation of activated CD4+T cells that co-express CD38, HLA-DR, and PD-1, which represented a deregulated effector memory phenotype and levels of these cells predicted disease progression. We showed that several mechanisms could explain the unique presence of these cells in HIV-1 infection. Together our data show alterations to innate and adaptive arms of cellular immunity, and may aid the development of pharmacological interventions. Below are the more salient findings that make up the body of this thesis. It is important to note that we observed no differences between HIV-1 subtype A or D infection in any of the parameters we have assessed.

PBMC processing and analysis in resource limited settings:

- High quality PBMCs can be reproducibly prepared and stored in a resource limited setting.
- Lymphocyte lineages cryopreserved well except for B cells where reductions were observed in both HIV-1 infected and uninfected individuals.
- Quality indicators for real-time performance assessment included: time from venipuncture to cryopreservation, processing time, yield from blood, and viability.
- Post thaw viability and yield are easy ways to monitor the integrity of cryopreserved specimens, while ICS provides a sufficient way to assess function.
- PBMC stored >3 years are well preserved for HIV-1 negative and positive patients.

NK cells in HIV-1 infected Ugandans

- NK cell counts correlate to CD4+ T cell counts but not viral load in chronic HIV-1 infection.
- HIV-1 alters NK cell subset distribution with a redistribution to $CD56^{neg}CD16^{+}$ NK cells and reduction in $CD56^{dim}CD16^{+/-}$ NK cells
- HIV-1 alters NK cell receptor expression with decreased inhibitory receptors KIR2DL1, NKG2A, and CD161 and decreased levels of activating receptor NKp30.
- HIV-1 increases NK cell function in unstimulated and K562-stimulated conditions and $CD56^{neg}CD16^{+}$ NK cells are not anergic.
- Changes in NK cell phenotype and function correlate with CD4 T cell loss. For example, CD4 counts were negatively correlated to function in $CD56^{bright}$ NK cells.

- HIV-1 infection is associated with increased KIR3DL1+ $CD56^{dim}CD16^{+/-}$ NK cells and decreased KIR2DL1+ $CD56^{dim}CD16^{+/-}$ NK cells in patients carrying their respective HLA ligands.
- The frequency of KIR3DL1+ $CD56^{dim}CD16^{+/-}$ NK cells is directly proportional to HIV-1 load in HLA-B Bw4 carriers.
- Chronic HIV-1 infection is associated with increased polyfunctional $CD56^{dim}CD16^{+/-}$ NK cells.
- Higher responsiveness in KIR3DL1+ $CD56^{dim}CD16^{+/-}$ NK cells in HIV-1-infected HLA-B Bw4 carriers than in noncarriers.

T cell activation in chronically HIV-1 infected Ugandans

- Frequency of CD4+ T cells that co-express CD38, HLA-DR, and PD-1 is increased in chronic untreated HIV-1 infection.
- CD4+ T cells that co-express CD38, HLA-DR, and PD-1 also exhibit an effector memory phenotype (CCR7-, CD28+ and CD45RO+) and higher Ki67 expression.
- CD38+HLA-DR+PD-1+ CD4+ T cell frequency is inversely proportional to T_{regs} frequency indicating a deregulated effector memory phenotype (CD4+ T_{DEM} cells).
- CD4+ T_{DEM} cell levels are directly proportional to viral load, inversely proportional to CD4 T cell absolute counts and predict progression to AIDS defining events.
- CD4+ T_{DEM} cell levels are directly proportional to sCD14 and IL-6, indicating a role for microbial translocation and innate activation in expansion of this subset.
- CD4+ T_{DEM} cells are generated *in vitro* to a variety of antigen stimulations and are enriched for viral (CMV and HIV-1) specific cells stimulated *ex vivo* using ICS to measure IFN-γ and TNFα production.
- Direct *ex vivo* CD4+ T_{DEM} cells display a TCR Vβ distribution similar to the overall CD4 compartment, suggesting these cells are specific for a diverse array of antigens.

Future Considerations: The above work represents advancement to our current understanding of the immune dysfunction observed in chronic HIV-1 infection. We would like to follow up this work in a number of areas:

1. PBMC function using cryopreserved samples is preserved, but the extent of preservation of antigen specific cells is not known.
2. NK cell phenotype and function are anecdotally compromised in cryopreservation, although we have never experienced problems with this. A more concentrated and focused effort should be made to address this.
3. $CD56^{neg}CD16^{+}$ NK cells should be characterized more in depth regarding their phenotype and function in HIV-1 chronic infection, and after ART.
4. KIR3DL1+ NK cells should be explored in early acute infection, and before and after ART initiation to examine if this population is responding to HIV-1 virus and determine role if any in viral control.
5. CD4+ T_{DEM} cells should be monitored before and after the initiation of ART to determine if this population restores after treatment initiation.
6. Mechanisms behind development of CD4+ T_{DEM} cells should be further explored, and role of cell associated viral load and bystander activation further characterized, particularly in early acute infection where little information regarding the formation of these cells exists.

7. Links between the adaptive and innate immune compartments, particularly between CD4+ T cells and NK cells should be explored to understand how these cells interact to control viral infections, and delineate common factors that generate elevated pathologically activated states.

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Figure 12. Meeting in Chiang Mai, Thailand (Nov. 11, 2009). Pictured from left to right: Michael A. Eller, Jeffrey R. Currier, Merlin L. Robb, Fred Wabwire-Mangen, and Johan K. Sandberg

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