ROLE OF INNATE CELLULAR IMMUNITY IN THE IMMUNOPATHOGENESIS OF HIV-1 INFECTION IN UGANDA

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Role of Innate Cellular Immunity in the Immunopathogenesis of HIV-1 infection in Uganda

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“Today it's me
Tomorrow someone else
It's me and you
We've got to stand up and fight
We'll shed a light in the fight against AIDS
Let's come on out
Let's stand together and fight AIDS”

Lyrics to ‘Alone and Frightened’ by Philly Bongoley Lutaaya

*It is* the glory of God to conceal a thing: but the honor of kings *is* to search out a matter.
Proverbs 25:2, The Bible, King James Version
ABSTRACT
The global epidemic of HIV has resulted in more than 34 million deaths and currently 36.9 million HIV-infected people worldwide. Uganda has an HIV prevalence of 7.3%, equating to about 1.6 million people. This has placed enormous pressure on the social, economic and medical structure of society. HIV is phylogenetically diverse, with HIV-1 subtypes A, B and C accounting for ≥70% of infections globally. While HIV-1 subtype C confers the worst prognosis for a patient, it is closely followed by subtype D which, together with subtype A, accounts for ≥90% of HIV infections in Uganda. HIV infection is associated with rapid viral replication, concomitant inflammation and immune activation, and massive CD4 T cell loss, which all together contribute to morbidity and eventual death. Although antiretroviral therapy lowers viral load and improves CD4 T cell recovery in chronic infection, it does not fully eliminate chronic immune activation nor restore immune function, resulting in non-AIDS related morbidity. Additionally, despite great effort, a preventive or therapeutic vaccine is yet to be developed.

Studies in chronic untreated HIV-1 infection may shed more light on correlates of immune protection that may be utilized to develop effective preventive or therapeutic vaccines or drugs. The innate immune system, as the first to encounter the HIV virus upon exposure and infection may be critical in directing immune responses that can prevent, attenuate or cure infection. In this thesis I aimed to study the role of the innate cellular immunity in the immunopathogenesis of HIV-1 subtype A and D infection in Uganda. In Paper I, using whole blood from healthy blood bank donors, we established normal lymphocyte reference ranges for Ugandans and showed demographic differences that may influence immune responses to disease and vaccination. Additionally, utilizing cryopreserved peripheral mononuclear blood cells from chronic untreated HIV-1 infected persons we studied the phenotypes and function of natural killer cells, unconventional T cells and regulatory T cells plus their roles in HIV-1 infection (Papers II-IV). Here we found both HIV-associated immune dysregulation of multiple cellular subsets and expansion of a previously little described innate-like terminally differentiated CD8 T cell subset. Furthermore, in Paper V we describe demographic differences in biomarkers of inflammation that not only associate with disease progression, but also expand our knowledge of HIV-related gut dysbiosis. Thus, the data presented here provides more insight into HIV-driven immune dysfunction, subtype-related immunopathogenesis, and demographic differences that add to the body of knowledge concerning HIV infection.
LIST OF SCIENTIFIC PAPERS


### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cell cytotoxicity</td>
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<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
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<td>ART</td>
<td>Anti-retroviral therapy</td>
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<td>APC</td>
<td>Antigen presenting cells</td>
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<tr>
<td>CCR5</td>
<td>CC chemokine receptor 5</td>
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<tr>
<td>CCR7</td>
<td>CC chemokine receptor 7</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
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<tr>
<td>CXCR4</td>
<td>Cysteine X cysteine receptor 4</td>
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<tr>
<td>DAMP</td>
<td>Damage-associated molecular patterns</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>Eomes</td>
<td>Eomesodermin</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<tr>
<td>FOXP3</td>
<td>Forkhead box protein 3</td>
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<tr>
<td>GrzB</td>
<td>Granzyme B</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
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<tr>
<td>HLA-DR</td>
<td>Human leukocyte antigen – antigen D related</td>
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<tr>
<td>IFABP</td>
<td>Intestinal fatty acid binding protein</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>iNKT</td>
<td>Invariant natural killer T cell</td>
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<tr>
<td>KIR</td>
<td>Killer-cell immunoglobulin-like receptor</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MAIT</td>
<td>Mucosa-associated invariant T cell</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
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<td>NCR</td>
<td>Natural cytotoxicity receptor</td>
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<td>NK</td>
<td>Natural killer</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PAMP</td>
<td>Pathogen associated molecular patterns</td>
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<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
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<tr>
<td>PD-1</td>
<td>Programmed death receptor-1</td>
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<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
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<tr>
<td>SEB</td>
<td>Staphylococcal enterotoxin B</td>
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<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<td>Treg</td>
<td>Regulatory T cells</td>
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<td>T-bet</td>
<td>T box transcription factor</td>
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1 HIV-1/AIDS

1.1 Overview

It is now close to 35 years since the first cases of the acquired immune deficiency syndrome (AIDS) were first described in five homosexual men in the United States \(^1\). In the beginning, doctors from different parts of the world described patients presenting with aggressive and rare infections and cancers that resisted most forms of medication. Within a few years it had become clear that these were a collection of syndromes that resulted from generalized immune deficiency arising from infection with a yet to be identified agent. In 1983, Francoise Barré-Sinoussi and Luc Montagnier identified the causative agent of AIDS \(^2\) that was later described to preferentially infect \(^3,4\) and cause loss of CD4 T cells \(^5\), and was named human immunodeficiency virus (HIV) \(^6\).

Uganda is an East African country with a population of over 34 million people \(^7\), of whom approximately 1.6 million are living with HIV \(^8\). Here, the first cases of AIDS were noticed in the south western district of Rakai (Figure 1) in 1982, and fully described by Serwadda et al. in 1985 \(^9\). These were atypical in that rather than the generalized lymphadenopathy and aggressive Kaposi’s Sarcoma seen in patients in western countries, Ugandans presented with extreme weight loss and diarrhoea occasioning the local name ‘Slim disease’, but with the immune deficiency recognised by anergy to most skin tests. Epidemiological comparisons later showed that this was the presentation of AIDS among Africans \(^10\). In 1986, Clavel and co-workers isolated another retrovirus from West African patients with symptoms similar to Slim disease that was different from the HIV virus \(^11\); through molecular cloning it was shown to be related to HIV, but with a different genomic sequence \(^12\). This led to the designation of the virus causing most infections globally as HIV-1, and the less prevalent West African type HIV-2.
1.2 HIV-1 epidemiology

By all accounts HIV/AIDS is still a major global disease; according to the World Health Organization (WHO), more than 34 million people had died while 36.9 million people were living with HIV at the end of 2014, 2 million of whom contracted it the same year. Sub-Saharan Africa, is host to approximately 70% of all people living with HIV, and 70% of all new infections globally. One reason this region is disproportionately affected may be because HIV originated here, with simian immunodeficiency virus (SIV, a non-human primate retrovirus) having crossed into humans around 1920. Identification of AIDS as a new disease was quickly followed by delineation of routes of transmission that include sexual intercourse, sharing of needles, transfusion with contaminated blood and blood products, and from mother to child during pregnancy, delivery and breastfeeding. It was soon clear that this was a global epidemic, with clusters of infection in different parts of the world quickly translating into generalized epidemics with differing epidemiology and presentation. Luc Montagnier and Robert Gallo describe a period of rapid discoveries of different facets of the epidemic that included characterization of the viral structure and genome, discovery of sequence variation, definition of viral proteins, development of a blood test and identification of the first antiretroviral drugs.

It should be stressed that the epidemic was devastating to social, economic and medical structures in affected societies, particularly in Africa. Countries recovering from political and attendant economic turmoil were ill-placed to handle a generalized epidemic such as HIV. The essential workforce of 15-40 year olds were the most affected, leading to huge
losses in productivity, and disruption of family structure. The effects on the economy led to a cycle of poverty that increased risky behaviour such as prostitution and cross-generational sex, thus increasing the risk of acquiring the infection. Health systems were ill-equipped to handle the influx of severely ill patients, both by numbers and complexity of the disease. Amid all this it became clear that HIV infection in Africans, while sharing many similarities with that in the western world, had significant differences that accentuated the effects of the disease at the individual and population level.

1.3 HIV virology

Phylogenetic studies have shown that HIV, a retrovirus of the lentivirus family, originated from several zoonotic events between non-human primates (NHPs) and humans, in Cameroon and the Democratic Republic of Congo (reviewed extensively in 42, 43). The two major lineages of HIV, HIV-1 and HIV-2, originated from independent transmission events to humans, have multiple groups within each, and have undergone further rapid gene mutation and recombination in the human population due to extensive virus-host interaction (reviewed in 44). Based on sequence similarity HIV-1 strains are classified into groups M (which is responsible for the HIV pandemic), N, O and P. HIV-2 has groups A-H, and is largely restricted to West Africa. HIV-1 group M is subdivided into subtypes A, B, C, D, F, G, H, J and K, and is widely distributed throughout the world (Figure 2), particularly in Africa where subtypes A, C, D and CRF02-AG account for over 80% of infections (reviewed in 45). Globally, the most prevalent HIV subtypes are A, B, and C with subtype C most predominant. In addition, there are a large number of circulating recombinant forms (CRFs) and unique recombinant forms of HIV-1.
Advances in technology have enabled a thorough description of the HIV replication cycle (reviewed by 41). HIV-1 primarily binds the target cell through the CD4 receptor, and thus mainly infects CD4 T cells 4 but also monocytes and macrophages that express the CD4 receptor, albeit at lower levels 46 (reviewed in 47). This leads to a series of events that include binding a chemokine receptor as co-receptor, usually CCR5 or CXCR4, fusion of the viral membrane with the cell membrane and injection of the viral capsid into the cytosol (reviewed in 48-50). This is followed by reverse transcription of viral RNA into DNA, transport into the nucleus and integration of viral DNA into cell genome. Later viral mRNA is transcribed and transported out of the nucleus into the cytoplasm for translation into viral proteins that are assembled and released as immature virions at the cell surface, which then undergo post-release maturation. This cycle is repeated on a massive scale leading to establishment of HIV infection in cells and tissue within days of infection. Integration of viral DNA into the host genome is one of the hallmarks of HIV infection and leads to establishment of a viral reservoir through latency - a state of non-productive infection of a cell – and makes efforts to eradicate HIV reservoirs challenging (reviewed in 51).
1.4 HIV-1 disease, treatment and prevention

During acute infection the HIV virus infects and replicates in a cell over a period of up to 48 hours\textsuperscript{52}, producing over $10^4$ virions per cell\textsuperscript{53}. New virions and infected cells spread systemically from mucosal layers to lymphoid tissues\textsuperscript{54} (reviewed in\textsuperscript{55}). This is accompanied by an exponential increase in viral replication to peak viremia, that decreases to a steady-state viral load, also called set point viral load\textsuperscript{56}, which signifies the end of acute and beginning of chronic infection and is a prognostic marker for AIDS (reviewed in\textsuperscript{57}). Concomitantly with the first rise towards peak viremia there is rapid increase in innate proinflammatory cytokines such as interferon alpha (IFNα), interleukin-15 (IL-15), tumor necrosis factor (TNF), IFNγ, IL-6 and IL-18, and chemokines such as monocyte chemotactic protein (MCP-1) and inducible protein 10 (IP-10), to stimulate immune responses\textsuperscript{58}. This is followed by an increase in the immunoregulatory cytokine IL-10, in an attempt to dampen the immune response. Acute HIV infection is also characterized by a massive loss of CD4 T cells that begins within days of infection\textsuperscript{59}, particularly from the gut-associated lymphoid tissue\textsuperscript{60}, by NK cell-mediated killing\textsuperscript{61}, HIV-driven up-regulation of apoptotic molecules and subsequent apoptosis\textsuperscript{62-64} (reviewed in\textsuperscript{65}), and directly through DNA-dependent protein kinase\textsuperscript{66}; and increasing and persistent direct and bystander immune activation\textsuperscript{67,68}, known to be one of the major causes of HIV pathogenesis (reviewed in\textsuperscript{69,70}). This leads to a generalized immunodeficiency\textsuperscript{2-5} manifest particularly in low CD4 T cell counts\textsuperscript{3,4}, and impaired host immunity to a variety of opportunistic infections\textsuperscript{20}. The quantity of HIV-1 RNA in plasma, reflective of ongoing viral replication, has been found to correlate inversely with the CD4 T cell counts and directly correlates to HIV-1 disease\textsuperscript{71}. The quantitative competitive polymerase chain reaction (QC-PCR) is a sensitive and accurate assay for the monitoring of plasma viral load in patients at all stages of infection\textsuperscript{72}. Plasma viral load is now routinely used in the healthcare and clinical management of HIV infection.

The description of the HIV-1 replication cycle inspired scientists to develop antiretroviral drugs to block or counteract specific aspects of viral entry and replication. The first successful drug, azidothymidine (zidovudine), was associated with significant decreases in mortality and frequency of opportunistic infections\textsuperscript{38}, reduction in viral load, and improvement in CD4 T cell counts, but with significant toxicity\textsuperscript{73} and incomplete viral clearance. It quickly became clear that the virus mutates rapidly to become drug resistant\textsuperscript{74} and suggestions were made to develop a multiple-drug strategy\textsuperscript{75}. Approaches utilizing a
combination of at least three drugs, each targeting a different aspect of the replication cycle, revolutionized HIV treatment by lowering viral load to undetectable levels and markedly improving life expectancy. This is known as highly active antiretroviral therapy (HAART).

Further studies showed that initiating antiretroviral therapy (ART) earlier improves prognosis by preventing CD4 T cell loss, preserving gut and lymph node structure, as well as arresting aberrant immune activation. Thus the latest WHO treatment guidelines recommend ART for all HIV-1 infected persons with a CD4 T cell count ≤500 cells/mm³ of blood regardless of plasma viral load. In addition to improving quality of life of HIV-1 infected persons, the WHO has recommended the use of ART to reduce the risk of HIV transmission from an infected person to an uninfected individual, as well as pre-exposure prophylaxis (PrEP) to protect most at risk vulnerable populations from HIV acquisition. When taken within 72 hours of exposure ART protects against establishment of infection (termed post-exposure prophylaxis – PEP). ART is also successfully used to prevent transmission of HIV from mother to child (eMTCT).

Despite all this progress, there are still hurdles to overcome. Only about 50% of HIV-infected people eligible for ART are on treatment; by 2013, only 40% of HIV-infected Ugandans eligible for ART were on treatment, and yet this is one of the three countries contributing up to 48% of new infections in sub-Saharan Africa. It is noteworthy that immunopathogenesis differs according to infecting HIV type. Much fewer people infected with HIV-2 go on to develop AIDS when compared to HIV-1 (reviewed in ), despite up to 60% homology in amino acid sequence of the viral genome. Within HIV-1 group M, subtype C (globally most prevalent strain) and D (prevalent in East Africa) appear to confer worse prognosis as regards time to development of AIDS and death, CD4 T cell loss (reviewed in ) and treatment failure, yet it is not clear why. In addition, the immune system does not fully restore to its pre-HIV state even after long-term HAART, thus patients go on to develop non-AIDS related comorbidities that compromise their well-being and life expectancy (reviewed in ).

The ultimate solution would be to totally prevent/protect from acquisition of HIV. Behavioural interventions such as abstinence, being faithful to one partner and use of condoms, while having significant effects in reducing HIV incidence, can for various reasons
not totally eliminate transmission. Other strategies, such as medical male circumcision, while successful in preventing HIV acquisition in up to 66% of males\(^94\), have faced many challenges particularly due to resource constraints in the most affected countries\(^95\). Several HIV vaccine products and microbicides have been tested globally with little to no success (reviewed in \(^96\)\(^97\)). The exception is the RV144 phase III vaccine trial conducted in Thailand that showed a modest protection of 31% in the HIV-uninfected who received the vaccine\(^98\), though that was not good enough for public health purposes. The biggest challenge to these efforts has been lack of clarity on an immune correlate of protection\(^97\), although indepth analysis of data from the RV144 trial have begun to provide important clues\(^99\). It is increasingly believed that a single program may not prove effective, and that a combination of prevention strategies will be needed to radically reduce or eliminate HIV transmission in the human population\(^100\). In the meantime though, it is important that research on both treated and untreated HIV infection continues so as to identify ways of improving the prognosis of patients, and better understanding of correlates of protection that can guide vaccine design.
2 THE IMMUNE SYSTEM

2.1 Overview

The earth houses approximately 7.3 billion people as of 2015, in addition to other forms of life such as plants, animals and, importantly, microorganisms such as bacteria, viruses and fungi. This ecosystem requires that organisms form relationships so as to efficiently utilize resources, although some (relationships) end up being detrimental to one of the organisms. The immune system is a network of cells, tissues, chemicals or proteins designed to protect living organisms from microbial attack from microorganisms, while at the same time maintaining self-tolerance to avoid autoimmunity. It is bimodal in nature, having an innate and adaptive arm, with both cellular and humoral (antibody) components and, as a basic principle, distinguishes foreign antigen from self.

2.2 The innate immune system

The innate immune system is tasked with initial identification, protection and/or neutralization of antigen, as it recognizes a vast array of molecular patterns quickly and is highly developed in its ability to discriminate self from non-self. The simplest form of defense is the anatomical barrier, akin to a wall around a city, meant to define the perimeter of the individual and block foreign organisms from entering. The skin is the biggest of these organs but others include the mucosal surfaces in the mouth, airways, intestines, and genital tracts, which secrete antimicrobial peptides and chemicals plus mucus for defense. The innate immune system involves a complex recognition system where cells and tissues have germ line-encoded pattern recognition receptors (PRRs) that detect (1) foreign antigen in the form of pathogen associated molecular patterns (PAMPs) that are conserved through evolution of classes of bacteria, viruses, and some fungi and parasites, and (2) self-antigen in the form of stress molecules, misplaced proteins and chemicals, otherwise known as damage-associated molecular patterns (DAMPs) (reviewed in 102 103). The PRR-PAMP/DAMP complex activates intracellular signaling pathways that trigger proinflammatory and antimicrobial responses, involving molecules such as phosphatases, kinases and transcription factors (reviewed in 102). This in turn leads to rapid production of molecules such as cytokines and chemokines, and the expression of receptors and cell adhesion molecules required to present and neutralize the antigen through activation of the complement cascade, phagocytosis, cytotoxic killing and/or activation of the adaptive immune system. Note that some innate immune cells display memory generated by
epigenetic reprogramming during the initial response, and that results in a heightened response that is nonspecific and antigen independent \(^{104}\) – known as trained memory. Typical innate immune cells include granulocytes (neutrophils, eosinophils and basophils), antigen-presenting cells (APCs) such as dendritic cells, macrophages and innate lymphoid cells that include natural killer (NK) cells. NK cells are one of two major focuses of this thesis.

### 2.2.1 Natural killer cells

NK cells are innate lymphoid cells first described for their killing of tumor and virus-infected cells \(^{105,106}\). They develop from a common lymphoid progenitor into large granular lymphocytes constituting up to 15\% of all peripheral blood lymphocytes and have two main subsets, classified according to their expression levels of CD56 (neural cell adhesion molecule 1; NCAM): CD56\(^{\text{bright}}\) immature NK cell subset \(^{107}\) and the CD56\(^{\text{dim}}\) mature subset that constitutes about 10\% and 90\% of peripheral blood NK cells, respectively (reviewed in \(^{108-111}\)). CD56\(^{\text{bright}}\) NK cells mature and differentiate towards CD56\(^{\text{dim}}\) through a sequential loss and gain of multiple surface receptors \(^{112,113}\) in an IL-15 dependent manner, through the action of the transcription factors nuclear factor, interleukin 3 regulated (NFIL3), T-box expressed in T cells (T-bet) and eomesodermin (Eomes) (reviewed in \(^{114}\)). It should be noted that NK cells can also develop in both secondary lymphoid and non-lymphoid tissues (reviewed in \(^{115}\)), and that IL-15 is required for their maintenance \(^{116}\).

NK cells utilize germ-line encoded killer-cell immunoglobulin-like receptors (KIR) to recognize targets, some of which are activating and others inhibitory (reviewed in \(^{117}\)). Other activating NK receptors include natural cytotoxicity receptors (NCRs: NKp30, NKp44, NKp46, NKp80), C-type lectin receptors (NKG2C, NKG2D, NKG2E), adhesion molecules (DNAM-1), PRRs (Toll-like receptors – TLRs), while NKG2A is inhibitory. The major KIR ligands are MHC class I molecules, expressed by majority of cells in the host, and act to inhibit NK cell cytotoxicity. Absence of MHC class I – known as ‘missing self’ and often observed in virus-infected or cancerous cells – abrogates inhibition and thus results in NK cell activation (reviewed in \(^{118}\)). The expression of NK cell receptors is dependent on antigen experience \(^{119}\) and tissue environment \(^{120}\) and varies from cell to cell. The strength of activation induced by binding of receptors also varies from cell to cell, a feature that is utilized in NK cell education: inhibitory receptor binding to self MHC during development is
used to tune the NK cell against auto-reactivity while maintaining recognition and reactivity to infected or malignant cells (reviewed in 108, 109, 121). This is important, as NK cells are constitutively primed to kill targets without prior engagement through (1) exocytosis of cytolytic granules (perforin perforates the target cell and granzymes induce apoptosis) or (2) death receptors. They also modulate immune responses through the production of cytokines such as interferon gamma (IFNγ) and tumour necrosis factor (TNF), and β chemokines such as macrophage inflammatory protein (MIP)-1 alpha (MIP-1α; CCL3), MIP-1β (CCL4) and RANTES (CCL5) 122. These cytokines and chemokines are crucial in directing the adaptive immune response to viral infection 123, 124. On the other hand some chronic viral infections such as cytomegalovirus (CMV) epigenetically modulate NK cell expression of activating and inhibitory receptors, and signaling molecules, and thus alter the immune response 125, 126 (reviewed in 127). It should be noted that NK cells may directly recognize pathogens through PRRs and kill them 128.

NK cells also express a variety of cytokine and chemokine receptors and can thus be activated by the proinflammatory cytokines (IFNα, IL-2, IL-12, IL-15 and IL-18 129, 130, reviewed in 131) and ‘called’ to sites of infection and secondary lymphoid tissues by chemokines (CX3CL1, MIP-1α, MIP-1β, RANTES 132, 133), respectively, released by APCs. Through crosstalk between NK cells and DCs, this activation induces the mammalian target of rapamycin complex 1 (mTORC1) pathway 134 (up regulating metabolic processes) leading to release of the antiviral cytokine, like IFNγ, that stimulates an antiviral state and activates dendritic cells to mature and present viral antigens (reviewed in 123, 135, 136). In addition, activation of NK cells leads to generation of a long-lived memory NK cell pool 137, 138 104, 139 (reviewed in 140). Furthermore, IFNγ from NK cells mobilizes T cells to the appropriate areas of the lymph node for activation 141.

CD56dim NK cells express FcγRIIIa, the activating low affinity type I Fc receptor (CD16) whose ligation to an antigen specific IgG that then binds antigen on target cell results in antibody-dependent cellular cytotoxicity (ADCC 142, 143). CD56bright NK cells do not express this receptor. In chronic viral infections a third NK cell subset, the CD56neg CD16+ NK cells, barely visible in the peripheral blood of healthy persons, expand 144. This subset is believed to represent exhausted CD56dim NK cells although the direct subset relationships and mechanisms involved are still not firmly established. Interestingly, although CD56neg NK
cells are less functional than CD56<sup>dim</sup> NK cells, they retain the ability to express chemokines. It should be noted that in vivo activation of NK cells involves ligation of multiple of the aforementioned receptors, both activating and inhibitory plus chemokine receptors, adhesion molecules and TLRs, and requires a stronger activating than inhibitory signal to occur.

### 2.3 The adaptive immune system

The adaptive immune response is delayed compared to the innate immune response and is triggered by recognition of antigen presented on major histocompatibility complex (MHC) receptors by APCs or target cells (reviewed in). Secondary stimuli that amplify the signal include either ligation of costimulatory receptors on T cells with their ligands on APCs, or cytokine activation, leading to activation, differentiation and proliferation of antigen-specific effector cells (reviewed in), and establishment of a life-long memory pool of cells that will respond very quickly and strongly to subsequent exposure. The adaptive immune system consists of two major cell types: T cells which develop in the thymus and B cells that develop in the bone marrow. T cells use a transmembrane protein T cell receptor (TCR) while B cells use a transmembrane B cell receptor (BCR) to recognize cognate antigen. Engagement of these receptors triggers intracellular signaling pathways and transcription factors that activate gene expression leading to maturation, differentiation and proliferation of the naïve cell. This process also leads to down-stream activation of effector programs in T cells with production of cytokines and chemokines, and killing of target cells. In B cells the major effector response is production of antigen-specific antibodies. The specificity of adaptive effector cells is dependent on gene-rearrangement by somatic recombination of the V, D and J segments of the β chain of the T cell receptor (reviewed in), and the heavy and light chains of the B cell immunoglobulin (B cell receptor) to create millions of unique possibilities of cell receptor specificities needed to detect a variety of potential antigens. Thus, the adaptive antimicrobial response is tailored towards a specific pathogen. T cells are a major focus of this thesis.

#### 2.3.1 Conventional T cells

Naïve T cells are generated in the thymus and circulate between secondary lymphoid tissues and the periphery on the lookout for cognate antigen. They express the TCR in association with two other membrane-spanning receptors, the CD3 – a signal transduction complex...
universally expressed on T cells – and glycoprotein co-receptor that can either be CD4 or CD8. CD4 and CD8 receptors bind extracellular portions of MHC class II or MHC class I respectively, recruit tyrosine kinases to the TCR complex and thus amplify the signal. MHC class I molecules present short peptides derived and processed from intracellular pathogens or stress molecules while MHC class II presents longer peptides processed from exogenous pathogens. The formation of CD3-TCR-CD4 or CD8 complex with cognate peptide-MHC complex presented by APCs leads to phosphorylation of the intracellular domains of the TCR/CD3 complex and activation of kinases such as SYK, LCK and ZAP70 that lead to downstream signaling and activation of transcription factors that result in activation of the T cell. This process is energy intensive and requires upregulation of metabolism to support the increased demand. It primes the T cell for differentiation into an effector cell in an IL-2 dependent manner leading to clonal expansion and differential expression of chemokine and homing receptors that target cells in and out of peripheral compartments and secondary lymphatics. Once the infection has been cleared, the pool of effector cells contracts leaving some few that differentiate into long-lived memory cells that will be called upon on secondary challenge or antigen re-encounter. Naïve T cells can also proliferate by way of homeostasis in response to the cytokines IL-7 and IL-15 (reviewed in 154) with the latter being important for maintenance of effector T cells, thus ensuring a continuous supply even when the thymus contracts with age.

T cell differentiation phenotypes are described by the expression patterns of multiple molecules such as cytokine, chemokine, integrin, homing and adhesion receptors, and are important in denoting localization and function of a particular cell (reviewed in 156). CD4 and CD8 T cells can be categorized according to the surface expression of CD45RA and CCR7 (reviewed in 158-160), a receptor-linked protein tyrosine phosphatase crucial for signal transduction and thus cell activation (reviewed in 161), and a CC-chemokine receptor necessary for the homing of T cells to lymphoid organs and tissue, and motility therein, in response to chemokines CCL19 and CCL21 (reviewed in 141, 162), respectively. Naïve T cells are CD45RA⁺CCR7⁺, central memory T cells (TCM) are CD45RA⁻CCR7⁺, effector memory T cells (TEM) are CD45RA⁻CCR7⁻, terminally differentiated effector memory T cells (TEMRA) are CD45RA⁻CCR7⁻, while tissue-resident memory T cells (TRM) are CD45RA⁻CCR7⁻ that are CD69⁺/CD103⁻/+ depending on location (reviewed in 157). The T cell costimulatory molecule, Traf-linked tumor necrosis factor receptor family member CD27 is important for the generation of memory T cells (reviewed in 163, 164) as it amplifies
proliferation and enhances cell survival of antigen-specific T cells. It is therefore, together with CD45RA, also used to classify T cell subsets.

It should be noted that the chemokines CCL3, CCL4 and CCL5 are important for recruiting T cells through ligands such as CCR5 into lymph nodes draining sites of inflammation for antigen-specific activation and differentiation into memory T cells \(^{165,166}\) (reviewed in \(^{156}\)). Beyond recruitment, CCR5 ligation influences T cell production of IL-2 and its dependent function inclusive of T cell proliferation \(^{167}\). TCM circulate between blood and lymphoid tissue, TEM between blood and peripheral tissue, TRM remain in specific tissues, while TEMRA are found mainly in circulation. Naïve CD4 T cells, once activated in lymphoid tissue, differentiate into TCM, then into TEM that are thought to differentiate into TRM in mucosal, peripheral and lymphoid tissue by homeostasis or upon secondary stimulation. On the other hand, naïve CD8 T cells, once activated in lymphoid tissue, are thought to differentiate directly into either TEMRA or TEM, with the latter differentiating into TRM in mucosal, peripheral and lymphoid tissue by homeostasis or upon secondary stimulation. This provides a lifelong pool of polyfunctional antigen-specific rapid responders to infection. CD8 T cells have recently been shown to differentiate, in the elderly, into a new subset of cells retaining a naïve phenotype but as polyfunctional as memory T cells (T\(_{MNP}\) cells) in response to persistent infections \(^{168}\). It should be noted that terminally differentiated cells undergo replicative senescence whereby they cease to proliferate but express high amounts of proinflammatory cytokines \(^{169}\) due to the shortening of telomeres, in a bid to protect the host from cells that become increasingly dysfunctional (reviewed in \(^{170}\)).

CD4 T cells are primarily helpers as they direct adaptive immune responses by activating other immune cells; influence innate immune responses; and perform antiviral functions through production of cytokines such as IFN\(\gamma\) and TNF, in addition to direct cytotoxic killing through perforin and granzymes (reviewed in \(^{171}\)). The nature and load of pathogen CD4 T cells encounter, APC involved, and resulting inflammatory milieu stimulates distinct transcription factors, affects the phenotype and function the cells adopt \(^{172}\) and determines the subset a naïve CD4 T cell differentiates into (reviewed in \(^{171,173}\)). Type 1 helper T (T\(_{H1}\), transcription factor T-bet) cells develop in the presence of high levels of pro-inflammatory cytokines such as IFN\(\gamma\), IL-12 and type I IFNs and primarily produce IFN\(\gamma\). T\(_{H2}\) cells develop in the presence of IL-4 and primarily produce IL-4, IL-5 and IL-10 (transcription factor
GATA-3). TH17 cells develop in the presence of IL-6 and TGFβ and primarily produce IL-10, IL-17 and IL-21 (transcription factor RORγt). T FH (T follicular helper, transcription factor BCL-6) cells develop within B cell follicles in the presence of IL-6 and IL-21 and primarily produce IL-4 and IL-21. Regulatory T cells (Tregs, transcription factor FOXP3) develop in the presence of TGFβ but without IFNγ, IL-4 and IL-6, and primarily produce the anti-inflammatory cytokines IL-10, TGFβ and IL-35.

Memory CD4 T cells enhance immune responses on secondary challenge or reinfection by rapidly activating innate immune responses through their production of proinflammatory cytokines, chemokine-driven recruitment of antigen-specific cells, providing help to B cells and CD8 T cells, and as effectors. For example, IL-2 from CD4 T cells helps tune NK cell proliferation, IFNγ secretion and recognition of MHC class I-devoid cells (reviewed in 174). On the other hand, licensed NK cells activate and augment CD4 T cell differentiation, proliferation and function through production of proinflammatory cytokines 175 and co-stimulation 176 but can also kill infected and stressed T cells when they upregulate death receptors or ligands for NK cell activating receptors (reviewed in 177).

Tregs are specialized CD4 T cells that counteract inflammatory processes to dampen immune responses and thus protect the host from immune-mediated pathology, although this sometimes leads to the persistence of pathogens (reviewed in 178, 179). Tregs also protect against autoimmunity as many of them express TCRs with high affinity for self-antigen. As such Tregs can be found in various tissues, both lymphoid and non-lymphoid, and particularly in sites of inflammation. There are two major subsets of Tregs: those that exit the thymus as Tregs (or natural Tregs; nTregs) and those that differentiate from CD4 T cells in the periphery in the presence of appropriate cytokines and antigen (peripheral/induced Tregs; pTregs/iTregs). Central Tregs (cTregs) circulate between blood and secondary lymphoid tissue where they actively block the priming of naïve CD4 T cells if self-antigen is present through the ligation of inhibitory receptors on dendritic cells. Homeostatic maintenance of Tregs is IL-2- and FOXP3-dependent, thus they highly express the high affinity IL-2 receptor α CD25 while only low amounts of the IL-7 receptor CD127 180. Once activated, cTregs differentiate into effector Tregs (eTregs) that are resident in non-lymphoid tissue and can be recruited to sites of inflammation where they act to counter inflammatory processes through production of cytokines like IL-10, activation of apoptosis, or interruption of metabolic
pathways. They express multiple homing and adhesion receptors/ligands that facilitate their entry into the periphery, and are maintained through IL-33, in addition to TCR and costimulatory molecule signals. It should be noted that proinflammatory cytokines such as IL-1 and type-1 IFN impede Treg function. On the other hand, Tregs can dampen NK cell function (reviewed in 174) and T cell expansion 181 by limiting the IL-2 available to them.

T-bet and Eomes are the transcription factors governing the differentiation of naïve CD8 T cells to effector cells. Akin to NK cells, CD8 T cells are primarily cytotoxic, killing virus-infected, malignant, and stressed cells through perforin and granzyme B (GrzB), but also produce cytokines. They require CD4 T cell help to function optimally (reviewed in 171, 182) Through the binding of CD40L on CD4 T cells to CD40 on DC, they license the DC to efficiently prime CD8 T cells to perform cytotoxic function 183. Antigen-stimulated naïve CD8 T cells that do not receive CD4 T cell help undergo activation-induced cell death when they encounter secondary stimulation. However, IL-15 expressed by licensed DCs can be sufficient to activate CD8 cytotoxic responses without CD4 T cell help. The cytokines IL-2 and IL-12 are necessary for antigen-specific CD8 T cell responses 184 as they stimulate clonal expansion and improved function. The need for IL-2 is tied to its effect on CD8 T cell metabolism and transcription through mTORC1, which affects differentiation into effector cells 185. IFNγ from CD4 T cells directs the recruitment and maintenance of tissue-resident memory CD8 T cells in a chemokine-dependent manner in response to mucosal infection. In addition, CD4 T cell derived IL-21 is necessary for the maintenance of effector CD8 T cell clones and activity in chronic viral infections, while IL-10 from regulatory T cells dampens the pro-inflammatory response and thus allows CD8 T cell memory maturation while protecting from exhaustion. CD4 T cells can also directly co-stimulate CD8 T cells through CD40 ligand – CD40 interactions, thus obviating the need for APC 186. Furthermore, direct T cell-to-T cell interactions during the priming phase in the lymph node contribute to the differentiation and generation of protective memory CD8 T cells 187. Thus dysregulation of CD4 T cell frequency, phenotype and function affects the quality of CD8 T cell function.

Activated T cells up-regulate a number of surface molecules including CD38 and HLA-DR, to mention but a few. CD38 is a transmembrane glycoprotein whose upregulation early during T-cell activation leads to changes in cell metabolism 188, cell-to-cell adhesion and
movement through the endothelial cell wall and cytokine production (reviewed in ). HLA-DR is an MHC class II receptor that is involved in antigen presentation, but that is also upregulated in response to activation. As earlier mentioned T cells require a co-stimulatory signal, in addition to the CD3-TCR-CD4/8 complex and cytokine signals. Some co-stimulatory receptors can deliver either activating or inhibitory signals depending on which ligand they bind (reviewed in ). For example, either of the ligands CD80 and CD86 on APCs can bind either of receptors CD28 or cytotoxic T lymphocyte antigen 4 (CTLA-4) on T cells, resulting in either an activation or inhibition signal respectively. Inhibition signals are utilized to modulate the activation signal, thus avoiding hyper activation whilst tuning cell differentiation and maturation. Another negative regulator of function is programmed death-1 (PD-1) and its ligand PDL-1. It should be noted that in the case of persistent immune activation, such as happens with chronic viral infections, T cells up-regulate a number of these negative regulators of function in a bid to counteract chronic stimulation: this up-regulation is associated with a state of exhaustion (that is, dysfunction seen in impaired proliferative ability and effector function).

2.4 Unconventional T cells

The description of the human immune system as bimodal is in a number of ways an oversimplification, as the discovery of cell types displaying a hybrid innate-adaptive character has increased (reviewed in ). In general these cells express surface receptors of restricted antigen specificity and constitutively display adaptive-like characteristics (for example memory), although highly specific cells displaying innate-like function (for example responding rapidly) have also been described (reviewed in ). Unconventional T cells include T cells displaying αβ TCRs that are semi-invariant and not MHC-restricted. Mucosal associated invariant T (MAIT) cells are a large innate-like T cell subset constituting up to 10% of T cells in peripheral blood, but are also found in liver and mucosal sites, and throughout the body. The semi-invariant TCR consists of a Vα7.2-Jα33 chain bound to an oligoclonal CDR3β-chain, which recognizes antigens presented on MR1 (reviewed in ). MR1-presented antigens are intermediates of the riboflavin biosynthesis pathway, thus these cells are important in fighting bacterial and some fungal infections. They can also be activated by cytokines such as IL-12 and IL-18 resulting in the production of IFNγ.
Invariant natural killer T (iNKT) cells are innate-like T cells whose invariant TCR recognizes lipid and glycolipid antigens (reviewed in 201) presented by the non-classical CD1d molecule (reviewed in 202). They constitute only up to 1% of peripheral blood T cells although they are found more abundantly in tissue. Three subsets of iNKT cells have been described, namely type I, type II and type Ia NKT based on the composition of the TCR and antigens they recognize (reviewed in 203); this thesis shall focus on type I, hereafter referred to as iNKT cells. The lipid α-galactosylceramide is the archetypical antigen for the iNKT cell TCR that is composed of a Vα24 and Jα18-containing α-chain bound to Vβ11-containing β-chain. Both self and foreign lipid antigens (particularly from bacteria) are recognized for thymic selection and tuning, and immune activation respectively. They constitutively express a number of activating NK receptors in addition to cytokine and chemokine receptors (such as CCR5 and CXCR4) that help them home to inflamed tissues, where they are found more abundantly. Like conventional T cells, they require IL-15 for maintenance and express either CD4, CD8 or none of them (double negative), although the majority express CD161 (reviewed in 204). Conversely, iNKT cells can be activated by either a TCR, cytokine (such as IL-12 and IL-18) or activating receptor signal 205. Activation results in rapid production of large amounts of both proinflammatory and immunoregulatory cytokines and chemokines (IFNγ, IL-4, IL-17, CCL3, CCL4, CCL5) and cytolysis (granzyme B and Fas-mediated apoptosis); thus iNKT cells are important in directing innate and adaptive immune responses to both PAMPs and DAMPs in a bidirectional manner (reviewed in 202 206).
3 HIV AND THE IMMUNE SYSTEM

3.1 Overview

The study of the interaction of HIV with the human immune system has dramatically accelerated our understanding of host-pathogen interactions. While there is a major immune bottleneck at the point of transmission \(^42\), once HIV infection occurs, it utilizes the immune system to cause an infection, rapidly replicate and establish latent viral reservoirs. This results in persistent inflammation and loss of CD4 T cells that leads to chronic activation, and eventual disease and death particularly in the absence of control of viraemia (Figure 3). Interestingly, even when viral replication is controlled to levels below detection by conventional tests, there is still residual replication that is suspected to cause the residual inflammation and activation that has been associated with non-AIDS morbidity. The following section delineates the contribution of different cell types to the immune control of HIV.

![Figure 3. A simplified timeline of untreated HIV infection.](image)

3.2 NK cells

At mucosal sites HIV-1 is recognized by epithelial cell TLRs triggering the release of cytokines such as IFN\(\alpha\) and chemokines such as MIP-1\(\alpha\) that attract and activate APCs \(^{124, 207}\). DCs process and present antigen, and release cytokines such as IL-12 and IL-18 that activate innate immune cells including NK cells and other effector populations that mediate antiviral activity (reviewed in \(^{208}\)).
HIV-1 accessory proteins Nef and Vpu inhibit antigen presentation in infected cells by downregulation of surface MHC class I molecules. This allows the virus to evade detection and cytolysis of the infected cell by CD8 T cells, while modulating NK cell recognition of infected cells. This can lead to NK cell production of IFNγ, TNF, β-chemokines and cytotoxic killing of autologous and allogeneic HIV-infected CD4 T cells through direct recognition and cytolysis or ADCC (reviewed in 213). The NK cell repertoire changes in response to HIV-1 infection, suggesting these cells become engaged in a systemic manner in fighting the virus (reviewed in 124). NK cells have been suggested to protect highly exposed seronegative injecting drug users and women at high risk of HIV infection from acquiring the infection. Certain KIR-HLA genotypes are associated with protection from HIV acquisition, low viraemia and slow progression to AIDS (reviewed in 221). Conversely, poor function in activated NK cells pre-infection and certain KIR-HLA combinations may be associated with higher risk of HIV acquisition. In rhesus macaques, SIV-specific CD4 T cells activate NK cells in an IL-2 dependent manner leading to better control of infection. HIV vaccine induced NK cell ADCC has been associated with reduced HIV infection risk in the RV144 trial, while high levels of passively acquired HIV-specific antibodies in infants were associated with reduced mortality risk.

HIV-1 uses many strategies to escape NK cell activity (reviewed in 228-230): Some HIV peptides can alter the binding ability of inhibitory KIRs to their HLA ligands towards greater NK cell inhibition. Infection is associated with altered expression of inhibitory and activating receptors (reviewed in 124) geared towards impairing cytolytic activity, and compromised ability to kill immature DCs and thus direct adaptive immune responses (reviewed in 238). NK cells display impaired secretion of cytokines and chemokines, impaired ADCC and respond poorly to cytokine activation. In chronic infection a dysregulated CD56NK cell subset expands (reviewed in 243). In vitro experiments also show impaired recruitment and activation of NK cells by dendritic cells exposed to complement-opsonized HIV. Conversely, HIV utilizes NK cells to compromise the immune response by, for example, killing of CD4 T cells through NKp44-NKp44L.
3.3 Conventional adaptive T cell responses

While it is not yet clear what role immune activation levels play in HIV acquisition\textsuperscript{222, 245, 246}, the chronic immune activation that occurs subsequent to infection, particularly in T cells, is a hallmark of HIV infection and predicts morbidity and mortality\textsuperscript{247, 248} (reviewed in\textsuperscript{249}). As mentioned previously, HIV mainly infects CD4 T cells, directly and indirectly leading to their death, and thus compromises host immune responses. The HIV envelope protein gp120 binds both the CD4 receptor and either of the chemokine receptors CCR5\textsuperscript{250} and CXCR4\textsuperscript{251} to facilitate its entry into a target cell (reviewed in\textsuperscript{49, 65}). The gut contains a large proportion of HIV susceptible CD4 T cells and the intense viral replication depletes CD4 T cells and compromises the gut barrier already in acute infection, leading to increased translocation of microbial products into the tissues and circulation (reviewed in\textsuperscript{252}). Gut damage is associated with alterations in the microbiome, both local and systemic inflammation that in turn activates immune cells, and recruitment of immune cells (through CCR5)\textsuperscript{253} thus increasing the pool of HIV targets. This, together with bystander activation\textsuperscript{254} is a major contributor to chronic immune activation\textsuperscript{67}. The importance of CD4 T cells in the immune response to HIV infection is underscored by the discovery that polyfunctional CD4 T cell responses were a correlate of protection against HIV acquisition in the RV144 trial\textsuperscript{255}.

CD4 T cell loss is associated with a marked increase in activated HIV-specific CD8 T cells that are poor at inhibiting HIV replication and predictive of disease progression\textsuperscript{256}. This may be due to selection of escape variants of virus\textsuperscript{257, 258} and results in an ‘arms-race’ between CD8 T cells and the virus\textsuperscript{259, 260} (reviewed in\textsuperscript{261}). Non-specific CD8 T cells also markedly expand: later on there is increased bystander activation\textsuperscript{262} and cycling of memory CD8 T cells that is associated with distortion of the structure of lymph nodes due to fibrosis\textsuperscript{68}. Furthermore, polyfunctional CD8 T cells are believed to be important for control of viral replication in acute HIV infection and for long-term non-progressive disease that occur in rare patients\textsuperscript{263-265}. Such efficient CD8 T cell responses also occur in elite controllers\textsuperscript{266, 267} (more resistant to apoptosis\textsuperscript{268}), and also display a memory response to viral rebound of SIV in pig-tailed macaques\textsuperscript{269}. 


Conversely, the HIV protein Tat activates CD8 T cells and may contribute to hyperactivation and exhaustion.

### 3.4 Invariant NKT cells and Tregs

Similarly, HIV can infect nTregs (reviewed in) and iNKT cells that express both CD4 and either of CCR5 or CXCR4 leading to their depletion. Interestingly, the transcription factor FOXP3 found in nTregs has been reported to have anti-HIV properties, which, in addition to resistance to apoptosis and altered expression of CD25, may partly explain the disparate results of Treg frequency, number and function in HIV infection (reviewed in). However, although they play a protective role in highly exposed seronegative individuals, Treg preservation does not lead to better control of chronic immune activation (reviewed in). On the other hand, iNKT cell depletion is associated with increased immune activation in SIV-infected macaques. In HIV the remaining iNKT cells express less cytokine and proliferate poorly to both cytokine and TCR stimulation in vitro experiments. HIV proteins Nef and Vpu modulate activation of iNKT cells by inhibiting CD1d cell surface expression and antigen presentation, suggesting that iNKT cells do play a role in HIV immune control. Interestingly, recent findings indicate that iNKT cells can detect HIV infection if Nef and Vpu activity is abolished.

### 3.5 Role of chronic immune activation

Persistent immune activation and inflammation is a hallmark of untreated HIV infection (reviewed in). Signs of chronic activation of immune cells in infected persons include elevated expression of activation markers such as CD38 and HLA-DR, and inhibitory receptors such as PD-1 and TIM-3 (reviewed in). Persistent activation is also accompanied by altered expression of cytokine and chemokine receptors, altered production of cytokines and chemokines plus aberrant killing ability, increased apoptosis, skewing of populations of cell subsets, impaired immune regulation, and immunosenescence (reviewed in). All these events and factors exhaust the immune response and predispose the
HIV-infected individual to opportunistic infections and cancers that are the typical causes of death. On the other hand, while ART preserves immune cell phenotype and function, gut integrity and lymph node structure, the residual low-level viral replication leads to residual immune activation that is suspected to be the cause of increased non-AIDS defining illnesses and accelerated aging. It is clear that it is important to prevent the establishment of a hyper activation state that begins within days of infection \(^{59}\) as natural SIV hosts (Sooty mangabeys and African green monkeys) arrest immune activation early and thus do not develop AIDS despite continuous viral replication (reviewed in \(^{69,249}\)). Innate immune mechanisms, as the earliest responses to HIV infection may be the key to this. Pig-tailed macaques infected with a replication- and disease- competent mutant of SIVmac239 also control virus and maintain health through both innate and adaptive mechanisms \(^{269}\). This thesis thus aimed to characterize innate cellular immunity in the immunopathogenesis of HIV infection in Uganda, in a bid to inform the development of better vaccines or therapies for HIV.
4. AIMS

HIV-1 infection continues to be a major cause of morbidity and mortality in sub-Saharan Africa. In Uganda, HIV-1 subtype A infection is associated with significantly slower progression to AIDS than subtype D infection. The main objective of this thesis was to significantly enhance our understanding of the role of innate cellular immunity in HIV-1 infection, and contribute to the body of knowledge leading to better vaccines and therapeutics. Thus the specific aims of this thesis were as follows:

Specific Aim 1: Establish normal reference ranges for lymphocyte subsets in Ugandans, including NK cells. Thus far, no study with a sufficiently large sample size and good representation of urban and rural Ugandan populations had been done. Country-specific reference values are very important for the design and interpretation of both basic studies and intervention trials where immunological parameters are of importance. (Paper I)

Specific Aim 2: Investigate the differential loss of regulatory subsets of T cells in HIV-1 subtype A and D infection. iNKT cells are innate-like T cells that direct innate and adaptive cell responses to an infection through their production of immunoregulatory and activating cytokines. Tregs modulate immune responses to infection to avoid immune activation-mediated pathology. We thus aimed to investigate if distribution of these cell subsets associated with the differential immunopathogenesis of subtype A and D infection. (Paper II)

Specific Aim 3: Determine the basis for the functional impairment of NK cells to cytokine stimulation in HIV-1 infected subjects. IL-12 and IL-18 are major cytokines involved in NK cell activation during an infection. The IFNγ NK cells release after cytokine stimulation is important not only for the anti-viral activity of this cytokine, but also the immunomodulatory effects on dendritic cell maturation that influence antigen presentation and T cell priming. (Paper III)

Specific Aim 4: Investigate the role of FcγRIIIA+ CD8 T cells in HIV-1 infection. CD16 is commonly expressed on innate immune cells like NK cells, monocytes and
neutrophils, and is involved in antibody-dependent cell-mediated cytotoxicity or phagocytosis. We sought to characterize the levels, characteristics and function of FcγRIIIA⁺ TCRαβ⁺ CD8 T cells and investigate their role in HIV-1 infection. (Paper IV)

Specific Aim 5: Assess the contribution of markers of inflammation to immune activation. Recent data shows that, unlike what is seen in western cohorts, viral load, but not T cell activation levels, was an independent predictor of disease progression in rural Ugandans. We sought to characterize innate soluble markers of inflammation and immune activation and identify predictors of HIV disease progression in this population. (Paper V)
5. METHODS

5.1 Flow Cytometry

Whole blood collected from participants in a long-term community-based cohort of HIV surveillance was processed and PBMCs cryopreserved according to previously established procedure. On the day of experimentation cryopreserved specimens were thawed and washed. Counts and viability were assessed on the Guava PCA (Guava Technologies, Hayward, CA, USA), using Guava ViaCount reagent. Standard flow cytometry phenotyping was performed as previously described. For assessment of transcription factors, cells were washed, permeabilized and fixed using an optimized kit (FoxP3 staining fix/perm, Ebiosciences) before intranuclear staining. Flow cytometry data were acquired with a BD LSR II instrument or a BD FACS Canto II instrument (BD Biosciences). Sorting was performed on a 4-laser special order BD FACS ARIA II SORP (BD Biosciences) contained in a biosafety cabinet. Clinical lymphocyte immunophenotyping was performed using the FACS MultiSET System and run on a FACSCalibur using the single platform Multi-test 4-color reagent in combination with TruCount tubes (BD Biosciences). The different panels studied are detailed in Table 1 and 2 below, although actual clone and manufacturer details can be found in the different papers.

Table 1: Flow panels used in thesis research

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Table 2: Flow panels used in thesis research

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5.2 Soluble factor analysis

Soluble factors were measured either by a human inflammatory cytokine bead array, custom multiplex cytokine array or by enzyme-linked immunosorbent assays (ELISA) commercial kits as per manufacturer’s instructions. Optical density (ELISA) was determined using a BioTek ELX800 plate reader and final concentrations were calculated from standard curves using KC4 software (BioTek, Winooski, VT). All samples were run in triplicate and mean values were used for data analysis. Details of soluble factors studied can be found in the different papers.

5.3 Gene expression analysis

Stained cells were sorted into wells (~1000 cells/well) containing 10 μl of reaction buffer (SuperScript III Reverse Transcriptase / Platinum Taq Mix, Cells Direct 2X Reaction Mix, Invitrogen). Reverse transcription and specific transcript amplification were performed using a thermocycler (Applied Biosystems Gene Amp PCR System 9700) as follows: 50°C for 15 min, 95°C for 2 min, then 95°C for 15 sec, 60°C for 30 sec for 18 cycles. The amplified cDNA was loaded into Biomark 96.96 Dynamic Array chips using the Nanoflex IFC controller (Fluidigm). This microfluidic platform was then used to conduct qPCR in nl reaction volumes. Threshold cycle (CT), as a measurement of relative fluorescence intensity, was extracted from the BioMark Real-Time PCR Analysis software. Amplified genes were qualified according to
whether they were efficiently amplified; the amplification was linear and was not affected by multiplexing \(^3\). Subsequent data analysis was performed using JMP software (version 10). Details of genes studied can be found in Paper IV.

5.4 ADCC assays

Measurement of ADCC was performed using the PanToxiLux (PTL) assay (OncoImmunin, Inc., Gaithersburg, MD, USA). Recombinant HIV-1 BaL gp120 from DAIDS, NIAID catalog #4961 and HIV-1 rgp 120 IIIB (CHO), catalog #1174 (obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) were used to coat targets CEM.NKRCCR5 cells. Optimum concentration used to coat target cells was determined for each gp120 through an 11-point titration starting with 20 μg/ml and serial diluting 2-fold. After coating target cells were labeled with TFL4 (OncoImmunin, Inc.), a fluorescent target-cell marker, washed and stained with viability dye LIVE/DEAD Fixable Aqua Dead Cell Stain (Life Technologies). Target cells were then resuspended together with sorted effector cell populations for an effector to target ratio of 30:1, in the presence of GrzB substrate (OncoImmunin, Inc.). After incubation Human Immunodeficiency Virus Immune Globulin (HIV-IG\(^\text{TM}\)) (North American Biologicals, Inc., Miami, FL, USA) was added to each well, and the plate incubated then washed. Cells were acquired on the LSRII (BD Bioscience) the same day. Fluorophores were detected using: a 488 nm 50 mW laser with 515/20 filters to detect GrzB substrate, a 406 nm 100 mW laser with 525/50 filters to detect Aqua L/D stain, and 640 nm 40 mW laser with 670/30 filters to detect TFL4 stain. Data were analyzed by using FlowJo 9.7.5 (Ashland, OR, USA).
6. RESULTS AND DISCUSSION

6.1 Lymphocyte subset distributions in Ugandan populations

The human immune system is composed of a complex network of cells that work in concert to protect the host from pathogen attack while avoiding immune-mediated pathology. It includes antigen-presenting cells such as dendritic cells and macrophages, granulocytes such as neutrophils, eosinophils and basophils, and lymphocytes such as T, B and NK cells. Hematology and clinical immunophenotyping are basic laboratory tests utilized routinely to guide clinical management of patients, with the latter being especially important in the case of HIV infection. In the context of research, baseline data on these parameters can help formulate research hypotheses, and is used to identify and characterize clinically relevant abnormalities. In addition, such data can be important in guiding choice of potential participants for clinical trials.

In Paper I we sought to establish normal lymphocyte reference ranges for Ugandans using blood collected from healthy blood bank donors. We found substantial sex-associated differences between women and men, with women having higher frequencies and counts for all lymphocytes with the exception of the frequencies of CD8 T cells and B cells that were similar, and frequencies and counts of NK cells that were higher in men (Paper I, Table 2). Of note, basophil counts positively associated with overall T cell counts.

Previous studies have shown that immune responses to viral infections differ between the sexes (reviewed in 308). In the context of HIV immunopathogenesis sex differences have been noted, with women displaying better clinical and virological outcomes in some data (reviewed in 317), and worse in others (reviewed in 317). Immune activation and inflammation both at the protein and soluble biomarker level also showed sex differences, with the latter study showing less reduction in inflammation and activation in the first year of ART for women, but with the former showing the reverse after years of ART. All this suggests that the sex differences probably i) stem from genetic and hormonal differences (reviewed in 317), ii) are revealed by data such as that presented in Paper I, and iii) have implications for disease outcomes. For example, a study by Weinberg et al revealed an increase of peripheral Tregs that suppressed immune responses in association with increases in progesterone. Sex differences also manifest in the pharmacokinetics and pharmacodynamics of drugs, including ART, so
much so that some drug regimens are prescribed differentially.\textsuperscript{319}

Although we show that basophil counts positively associated with T cell counts in both sexes, the association was stronger in women (\textbf{Paper I, Fig. 3 and 4}). Thus basophils may probably more strongly modulate immune responses\textsuperscript{320} and be one of the contributors to greater immune activation and inflammation seen in women (reviewed in \textsuperscript{321}), particularly those with a significant parasitic burden (reviewed in \textsuperscript{322, 323}).

Recent research also shows sex differences in metabolism\textsuperscript{324}; as nutrition influences metabolism, and metabolism influences immune responses\textsuperscript{151, 185}, this may contribute to the cellular differences seen here, and to differential HIV immunopathogenesis.

Furthermore, studies have shown sex differences in cellular function and phenotype\textsuperscript{325, 326}.

Interestingly, when we compared participants of two different cohorts, one urban and the other rural, to the semi urban blood bank donors, we found significant differences in both lymphocyte and hematological parameters, particularly in males. The three cohorts were disparate in terms of urbanicity but similar as regards heterogeneity of tribes (thus genetics) and altitude. Differences in cell subset proliferation and numbers have previously been recorded between ethnic groups in the same environment\textsuperscript{327, 328} in the context of HIV infection, within an ethnic group dispersed in different countries\textsuperscript{329} and within different locations in the same country\textsuperscript{330}. We hypothesize that nutrition, socio-economic status and composition of the intestinal microflora may contribute to the differences seen between these groups, although it is possible that subclinical conditions not evaluated while taking medical history could influence the results\textsuperscript{331, 332}. These results are relevant to HIV immunopathogenesis: a study conducted in semi urban districts of Uganda showed that antiretroviral therapy–naive HIV-positive adults with poor diet quality were more likely to be less educated and more socio-economically disadvantaged, and poor diet quality was positively associated with anemia and low CD4 counts in addition to predicting mortality\textsuperscript{333}. Another study conducted to investigate prevalence of intestinal parasites in two sub-ethnic groups living in the same area in Malaysia found that the statistically significant differential parasitic burden was influenced by socioeconomic factors\textsuperscript{334}. It is thus of interest that males revealed more location differences than women, as it points toward socioeconomic factors. Additionally, it emphasizes the need to not only include but
also endeavor to balance different demographic groups during data collection and analyses so as to pick up any important differences (reviewed in 335, 336) or at the very least validate the reference ranges using a sample of the population of interest 337. Thus further research with sufficient statistical power to detect how demographic differences may impact future clinical studies and trials are needed 312, 338 (reviewed in 321).

6.2 Natural killer cell phenotype and function in HIV-1 subtype D infection

HIV-1 subtype D infection is characterized by faster disease progression than subtype A infection in Ugandans, for yet to be defined reasons. Natural killer cells are important effectors of viral control in virus infections that direct both innate and adaptive immune responses. They are innately primed to respond to cells expressing stress ligands and/or missing MHC class I receptors. In order to function optimally, NK cells utilize a plethora of activating and inhibitory receptors that tune the threshold of activation thus protecting self against NK-mediated pathology. In HIV, they have been shown to help protect against acquisition, inhibit viral replication, and influence disease progression. In Paper III we investigated if NK cell phenotype and function influenced immunopathogenesis of subtype D infection in chronic untreated HIV-1 infected Ugandans.

In this cross sectional study, we found that CD56dim NK cells from HIV-infected persons produced less IFNγ compared to uninfected controls, when stimulated with the cytokines IL-12 and IL-18 (Paper III, Fig. 1b and Fig. 1c). This was in contrast to stimulation with MHC-null K562 cell line, which showed no difference. Surprisingly there was no difference in either cytolytic (perforin) or proliferation ability (Ki67) of the CD56dim NK cells seen as measured ex vivo, regardless of infection status. HIV-infected individuals had a lower representation of NKG2A+CD57+CD56dim NK cells than the healthy controls (Paper III, Fig. 1f and Fig. 1g); this subset was highly activated (HLA-DR; Paper III, Fig. 1h) and its frequency correlated directly with CD56dim NK cell IFNγ production in response to IL-12 and IL-18 stimulation (Paper III, Fig. 1i), but not K562 stimulation (Paper III, Fig. 1j). Of note, these changes in CD56dim NK cell phenotype and function were independent of CD4 T cell count and viral load. We concluded that these changes reflected an HIV-driven change in NK cell maturation that was accompanied by activation and poorer response to cytokine
stimulation.

IL-12 and IL-18 are innate cytokines that activate immune cells differentially to respond to pathogens. IL-12 stimulates NK cell production of IFNγ that skews naïve CD4 T cell differentiation to Th1 necessary for antiviral and antibacterial activity (reviewed in 171, 339), and enhances NK cell - CD4 T cell crosstalk 141, 175, 176 hence influencing adaptive immune responses. Cytokine stimulation also enhances NK cell cytotoxicity, both through perforin and Fas-Fas ligand pathways 340-342. Thus the defect in NK cell IFNγ production could lead to poorer immune responses to not only HIV but also opportunistic infections.

NK cells show defects in function in chronic infection, regardless of ART status 342. HIV-infection impairs the anti-fungal ability of NK cells by compromising NKp30 expression and impairing the perforin pathway 342. Stimulating the NK cells in vitro with recombinant human IL-12 restored NK cell function measured by perforin expression. However, this study did not assess the ability of these NK cells to produce IFNγ, despite there being conflicting results on whether the cytokine is important for anti-fungal activity 128, 343 (reviewed in 344). Thus, it is possible that Kyei et al would have found a defect in NK cell IFNγ production, and that we would have found a defect in NK cell perforin expression had we stimulated the NK cells ex vivo. Taken together it appears that NK cells display multiple defects in HIV infection that are only partially rescued by exogenous cytokine. This data is important particularly for the incidence and reactivation of fungal infections in the HIV-infected population in Uganda, as fungal diseases are quite common 345. Significantly immunosuppressed persons starting ART are at high risk for developing immune reconstitution inflammatory syndrome (IRIS), a condition in which patients deteriorate paradoxically despite both mycologic and virologic suppression 346. Meya et al showed an increase of activated CD56dim NK cells, CD4 T cells and proinflammatory monocytes in the cerebrospinal fluid of HIV-infected persons displaying cryptoccocal meningitis-IRIS 347. It is worth noting that patients who display high Th2 and Th17 responses but low proinflammatory cytokines pre-ART are predisposed to develop IRIS post-ART 348. Inflammasome activation leads to excessive inflammatory monocyte responses and ineffective T cells associated with mycobacterium tuberculosis-IRIS in HIV-TB coinfected persons 349. All these, together with the pathogen-associated skewing of NK
cell phenotype subsets described here and elsewhere reveal the importance of NK cells to the immune response.

In HIV infection, persistent inflammation and immune activation begins early in infection and is not resolved even when ART is started in acute infection. This suggests other drivers of inflammation and activation or low-level viral replication in local sites. Thus the defects we see here could have begun early in infection. Although the prevalence of hepatitis B, hepatitis C and syphilis were low, we did not evaluate these participants for other coinfections that may contribute to the immune dysregulation we see here. Nevertheless, the data presented has implications for the immune response to incident infections during HIV-1 infection and thus cumulatively lead to more rapid progression of HIV-1 subtype D infection.

6.3 Differential associations of interleukin 6 and intestinal fatty acid-binding protein

High levels of inflammation and immune activation begin in acute HIV infection and persist through chronic infection and are associated with morbidity and mortality in treated and untreated infection. In this study, we examined indices of immune activation and inflammation in Ugandans with chronic untreated HIV-1 infection. We found that IL-6, soluble CD14 (sCD14), soluble CD163 (sCD163), and catalase were elevated in HIV-1–infected study participants compared with uninfected individuals, although C-reactive protein (CRP), intestinal fatty acid binding protein (IFABP), IL-10, and neopterin did not differ significantly (Paper V, Table 1). A trend toward lower levels of IFABP was observed in HIV-positive participants compared with HIV-uninfected individuals (Paper V, Table 1). Viral load, T-cell activation and IL-6 levels associated with faster disease progression (Paper V, Fig. 1A) and with each other (Paper V, Fig. 1B). Surprisingly, even though IL-6 levels associated with CRP levels, IFABP levels associated neither with viral load nor with sCD14 (Paper V, Fig. 1B).

Our data on soluble biomarkers of inflammation from this rural African population differed from what has been described extensively in the literature, where neopterin and CRP predict HIV disease progression (reviewed in 356-358). In addition,
contrary to what is described in the literature (reviewed in 359), IFABP was lower in HIV-infected participants compared to uninfected ones. Sub-Saharan Africa is endemic for many infections, particularly enteric ones360, which may stimulate an inflammatory state independent of HIV-1 infection, so that HIV-infection does not dysregulate the gut above the other infections. Furthermore, accelerated enterocyte turnover facilitates expulsion of gut parasites361 that are endemic to sub-Saharan Africa (reviewed in 362); suggesting that lower IFABP levels in fast progressors compared to slow progressors may be a mark of dysregulated immune responses. Although sCD14 and IFABP have been used interchangeably as markers of microbial translocation, they were inversely correlated in our data, which is more in line with IFABP being a marker of slower progression in our cohort. This could also be reflective of independent biological processes in this cohort. Taken together, our data reveals differences in known soluble biomarkers of inflammation between cohorts from different regions that warrant more detailed investigation 363.

Consistent with other studies (reviewed in 356), markers of monocyte activation and inflammation were all increased in HIV-positive individuals compared with negative participants. In spite of this, CRP levels did not associate with viral load, suggesting multiple independent drivers of inflammation in this cohort. Again, the prevalence of Hepatitis B and C and active syphilis were low and did not differ between progression groups and uninfected, however, we did not test for other coinfections that could possibly contribute to the results described here (reviewed in 364). In the era of ART it is important to delineate which pathways contribute strongest to both AIDS and non-AIDS comorbidities (reviewed by 356), as this will guide future therapeutics and vaccines. A recent study conducted among rural Ugandans found that T cell activation was not an independent predictor of HIV disease progression87. Thus future work should systematically evaluate what biomarkers and factors associate with faster disease progression in African cohorts in comparison to western cohorts.

6.4 Subtype divergence and immunoregulatory T cell subsets

Invariant natural killer T cells direct innate and adaptive cell responses to an infection through their production of immunoregulatory and activating cytokines. Regulatory T
(Treg) cells modulate immune responses to infection through production of cytokines or directly by activating apoptotic and/or interruption of metabolic pathways. We thus aimed to investigate if distribution of these cell subsets associated with differential immunopathogenesis of subtype A and D infection. We found that HIV-1 subtype D infected Ugandans had a significantly lower level of iNKT cells than HIV uninfected Ugandans (Paper II, Fig. 1b). In contrast, subtype A subjects had a significantly lower level of Tregs than either subtype D infected or uninfected subjects, despite both subtypes showing Treg loss (Paper II, Fig. 1d). Interestingly, only in subtype A infection were correlations observed between total CD4 T cell function and iNKT cell frequency, when stimulated with SEB (positive association, Paper II, Fig. 1e, f and g) and CMV (inverse association, Paper II, Fig. 1h, i and j). The positive correlation of CD4 T cell function to iNKT cell levels held even when it was taken by single cytokine expression. There were no associations of iNKT cell levels to Treg levels, CD4 T cell absolute count or viral load in either subtype.

More significant iNKT cell loss in subtype D compared to subtype A infection despite being at the same stage of infection may contribute to the divergence in immunopathogenesis described in Uganda. Selective loss of immunoregulatory iNKT cells and dysfunction of remaining cells has previously been documented in HIV infection. Interestingly, a study of HIV-1 and the less virulent HIV-2 infection found loss of iNKT cells in HIV-1, HIV-1/2 and viraemic HIV-2 infected individuals compared to uninfected controls, suggesting that preservation of iNKT cells associated with less immunopathogenesis in this African cohort. iNKT cells were also more activated in viraemic compared to aviraemic HIV-2 infected individuals, and activation levels correlated with markers of disease progression. When these findings are taken together with preservation of iNKT cells in subtype A infection that associated with general polyfunctionality of CD4 T cells, it suggests that iNKT cell levels, and possibly function are important for protection against HIV disease. The loss of Tregs could mean less suppression of general immune responses and thus better clinical outcomes.
Of note, preservation/reconstitution of CD4 T cell ability to produce IL-2 has been associated with control of HIV viraemia \(^ {367}\), is a potential correlate of immune protection from HIV acquisition \(^ {255}\) and leads to improved NK cell function \(^ {368}\). Levels of polyfunctional CD4 T cells (IFN\(\gamma\) and IL-2 positive) associate inversely to viral load in HIV-TB coinfection, adding to the body of knowledge on the importance of these cells in the immunopathogenesis of HIV infection \(^ {369}\). While it is not clear why our findings were independent of CD4 T cell counts and viral load, it is possible that the defects we describe are established early in infection. Proulx et al show that iNKT cells are dysregulated quite early in infection, with differential influence of transmitted founder virus isolates on iNKT cell activity in female genital mucosa \(^ {282}\). Taken together, this data shows differences in immunoregulatory T cell levels, adaptive T cell responses and associations with viral subtype that may contribute to differential pathogenesis of HIV-1 subtype A and D infections.

6.5 Innate-like terminal effector CD8 T cells expand in HIV infection

CD8 T cells utilize a range of effector functions to combat viral infections, including cytolysis and effects mediated by cytokines and chemokines, utilizing antigen-specific T cell receptor recognition of antigen-bearing MHC in a restricted manner. However, CD8 T cells have been shown to develop innate-like characteristics in the context of chronic infection \(^ {370}\). In this study we hypothesized that late-stage differentiation of CD8 T cells may be associated with transcriptional changes that support innate-like effector functions in the T cell compartment. We found that indeed a \(\alpha\beta\) TCR bearing CD8 T cell population expressing the Fc\(\gamma\)RIIIA expands in chronic untreated HIV-1 infection, that they were highly activated (Paper IV, Fig. 1b-d) and persisted even with ART (Paper IV, Fig. 1g). The activation of this subset associated with plasma markers of HIV-driven systemic immune activation (Paper IV, Fig. 1i). Phenotypically, Fc\(\gamma\)RIIIA+ CD8 T cells were distinct from Fc\(\gamma\)RIIIA-CD8 T cells as they were terminally differentiated effector cells that expressed perforin and NKG2D expression while not expressing CD161 (Paper IV, Fig. 2a-c), and were thus less likely to be invariant T cells. The significant upregulation of the transcription factor Helios, at the protein level, distinguished Fc\(\gamma\)RIIIA+ CD8 T cells from Fc\(\gamma\)RIIIA- CD8 T cells and NK cells (Paper IV, Fig. 2d). Interestingly, when
we assessed KIR expression we found that FcγRIIIA+ CD8 T cells from HIV-infected persons had a similar expression pattern to NK cells, while they were intermediate between FcγRIIIA- CD8 T cells and NK cells in healthy donors (Paper IV, Fig. 2e). Transcriptomic analysis confirmed that the level of Helios gene IKZF2 and IL-7Rα gene transcription distinguished FcγRIIIA+ from FcγRIIIA- CD8 T cells and NK cells, while transcript levels of the NK cell-associated receptor NKp80 gene KLRF1 was similar between FcγRIIIA+ CD8 T cells and CD56dim NK cells (Paper IV, Fig. 3). Furthermore, the capacity of FcγRIIIA+ CD8 T cells to mediate HIV-1-specific ADCC was similar to that of FcγRIIIA+ NK cells on a per cell basis (Paper IV, Fig. 4c). Thus these late-stage effector T cells acquire FcγRIIIA expression in HIV-1 infected individuals and use it to mediate HIV-specific ADCC, a function normally associated with NK cells. Functional diversification of adaptive CD8 T cells may be important as therapeutic strategies evolve to include antibody-mediated mechanisms to eliminate HIV-1 reservoirs.

FcγRIIIA+ CD8 T cells expansion coincident with bulk CD8 T cell population expansion suggests a concomitant response to the chronic uncontrolled viral replication. In some murine models, bystander activated memory CD8 T cells can recognize and clear pathogens in a TCR-independent fashion (NKG2D and IFNγ plus GrzB). In another murine model, innate-like memory CD8 T cells respond to inflammasome-generated cytokine by promoting antimicrobial resistance in lymph nodes while inhibiting systemic spread. Jacomet et al describe a phenotypically similar population of ‘innate/memory-like’ CD8 T cells in both healthy adults and cord blood and hypothesize that they would expand in infection. Similarly, FcγRIIIA+ CD8 T cells maintained protein expression of T-bet and Eomes while downregulating CD127, which confirms that they are terminally differentiated effector cells. The FcγRIIIA+ CD8 T cells we describe adopt a KIR profile similar to NK cells in HIV-1 infected subjects, and their activation associates with systemic immune activation, suggesting that they expand in response to infection. NK cell KIR profiles have been found to influence HIV immunopathogenesis. Previously, Eller et al found expansion of polyfunctional KIR3DL1 CD56dim NK cells in chronic untreated HIV-1 infected Ugandans who expressed the appropriate HLA-B ligand. CD8 T cells from healthy donors have been shown to express single activating or inhibitory receptors independent of their HLA ligands and NK
cell inhibitory receptors\textsuperscript{377}. That FcγRIIIA+ CD8 T cells express multiple KIRs may be a feature of this subset, with the similarity to NK cells being a function of viral replication. The conditions \textit{in vivo} during HIV-1 infection thus seem to drive not only an expansion of these cells, but also expression of surface receptors beyond FcγRIIIA normally associated with NK cells.

At gene level, the FcγRIIIA+ CD8 T cells appear to have a transcriptional program intermediate between late-stage effector CD8 T cells lacking FcγRIIIA and CD56\textsuperscript{dim} NK cell expressing FcγRIIIA. The FcγRIIIA+ CD8 T cells displayed high expression of grzB and granulysin, maintained expression of IL-21R but with very low expression of IL-7Rα. It is thus possible that these effector cells are maintained by IL-7 independent mechanisms, such as by IL-21\textsuperscript{378,379}, as they remain stable over 12 months of ART. Consistent with upregulation of surface KIR expression in this subset in HIV infection, genes for other NK cell-associated receptors were also upregulated. Most interestingly, the KLRF1 gene, which encodes the activating receptor NKp80 was expressed at similar levels as in CD56\textsuperscript{dim} NK cells. NKp80 has recently been shown to associate with the development and maturation of fully functional NK cells in secondary lymphoid tissue in response to signals from DC in addition to cytokines such as IL-15\textsuperscript{380}. We hypothesize that NKp80 could be playing a similar role here. It is also possible that, like is seen with NK cells, FcγRIIIA+ CD8 T cells utilize NKp80 to foster mutual activation of the CD8 T cells and monocytes/macrophages for improved viral control\textsuperscript{381}.

In addition, the FcγRIIIA+ CD8 T cells displayed high expression of the transcription factor Helios both at the protein and gene levels, as well as a modestly lower expression of the transcription factor Eomes. At the protein level Eomes expression in the FcγRIIIA+ CD8 T cells appeared normal, which was consistent with previous studies\textsuperscript{374}. Helios has been shown to mark out T cells that have been activated and are proliferating\textsuperscript{382}. Possibly, then, the distinctive transcription factor pattern contributes to sustaining FcγRIIIA+ CD8 T cell expansion in HIV-1 infected individuals. It would also be interesting to establish if they can be activated and maintained by other means, such as cytokine and natural cytotoxicity receptors\textsuperscript{371,372,374}. 

38
The expansion of FcγRIIIA+ CD8 T cells we describe is reminiscent of CD8 T cells with a similar phenotype in HCV-infected patients. The chronic nature of these infections with rapid viral replication and high mutation rates, leads to selection of epitope immune escape variants. The FcγRIIIA+ CD8 T cells display a memory phenotype of expanded antigen-specific T cells but whose epitopes possibly mutated, and thus the new biological characteristics including additional mechanisms to trigger effector activity may be a way to repurpose this subset to contribute to the immune response. It is intriguing that in murine models innate-like memory CD8 T cells were important for the immune response to both viral and bacterial infections, particularly in the early response. More studies exploring the role of the FcγRIIIA+ CD8 T cells in reactivated and opportunistic infections in the context of HIV infection are warranted. The ADCC activity of these cells could perhaps be harnessed for HIV preventive, therapeutic and cure research.
7. CONCLUDING REMARKS

This thesis aimed to investigate what role innate immune cellular subsets play in the immunopathogenesis of chronic untreated HIV-1 infection in Ugandans. We also investigated cellular differences between subtype A and D infection in order to elucidate differential immunopathogenesis. A number of lymphocyte subsets were studied including NK cells, iNKT cells, regulatory T cell subsets and CD8 T cells exhibiting innate-like ADCC activity. Furthermore, the distribution of lymphocyte subsets and reference ranges were determined for the Ugandan population. We found altered and impaired phenotypes and functions in HIV infection consistent with HIV-driven immune dysregulation, although we could not rule out contributions from other coinfections not assessed in our studies. The impairments of NK cells and iNKT cells may contribute to the accelerated immunopathogenesis of subtype D infection. It is interesting that cytokines such as IL-12 and IL-18 can, at least hypothetically, activate most of the cell subsets described here, and yet we show impaired cytokine responses in NK cells. In addition, some CD8 T cells, in HIV infected donors, develop the capacity to perform ADCC and mediate HIV-specific immunity in this way thus possibly adding to the functional repertoire employed against HIV. Further work should be done to evaluate phenotype and function of these cells with different stimuli, particularly in the context of subtype D infection.
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