Influence of CD4+ cell types on HIV-1 infection
Heeregrave, E.J.

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1

GENERAL INTRODUCTION
Chapter 1

1. HIV-1

1.1 The epidemic
Human immunodeficiency virus type 1 (HIV-1) was in 1983 described as the causative agent of acquired immunodeficiency syndrome or AIDS. In 2008 an estimated 33.4 million people were infected with HIV-1 with 2.7 million new infections that year. Sub-Saharan Africa remains the most affected region accounting for 67% of HIV-1 infections worldwide and 91% of new infections amongst children. The widespread presence of malaria, tuberculosis and helminths forms an extra threat to HIV-1 infected individuals in this region.

HIV-1 is classified into three groups with the predominant M group responsible for the vast majority of infections. Viruses belonging to the N and O group have a very low prevalence and circulate in specific regions in Africa. Within the M group, HIV-1 consists of 9 subtypes (A-K) that have a distinct geographic distribution.

1.2 The virus
One HIV-1 particle contains two single-stranded RNA molecules. The genome of HIV-1 is 9.7 kb and consists of nine genes. The virus enters the cell through binding of the viral envelope to the CD4 molecule and an additional receptor; C-C chemokine receptor 5 (CCR5 or R5) and C-X-C chemokine receptor 4 (CXCR4 or X4) are the two predominant coreceptors utilized by HIV-1.

Following additional conformational changes, the fusion peptide protrudes into the cell membrane and the viral and cellular membranes are brought into close proximity allowing for membrane fusion and viral entry to occur. Reverse transcription of the viral genome is initiated in the cytoplasm before being transported to the nucleus, where the resulting cDNA is integrated into the cellular genome as proviral DNA.

Reverse transcription is an error-prone process due to the lack of proofreading activity of the reverse transcriptase enzyme which leads to a high error rate, estimated at one mutation per generated genome. This combined with the estimated $10^{10}$ virions produced each day within an infected individual and the high rate of recombination provides for a high overall mutation rate. The resulting swarm of closely related but distinct virus variants is termed the 'quasispecies'. An estimated 0.1% of these virions are infectious virus particles. This high level of viral diversity can reach 10% within the envelope gene within a patient. This enables HIV-1 to rapidly evolve in response to immune pressure and antiretroviral therapy ultimately allowing for escape viruses to quickly emerge.

1.3 Disease course
Upon acute HIV-1 infection the virus vigorously replicates and infects multiple tissues within the infected individual, but has the most devastating effect in the gut where the highest proportion of target cells reside. These are the CD4+ lymphocytes, of which 80-90% is depleted during the first weeks of infection. This loss is reflected by a decline in
cell numbers in the peripheral blood where viral load can reach 10 million copies per ml. Coinciding with the induction of an immune response following infection (1-3 months), viral load declines and CD4+ T cell loss is partially restored. Gut-associated lymphocyte cell numbers remain relatively low and only partially recover. The chronic stage of the infection lasts on average between 2 to 15 years and is in a normal progressing individual characterized by slow CD4 decline and an increase in plasma viral load. The final stage of infection is termed AIDS where people usually succumb to opportunistic infections due to a severely weakened immune system. Some individuals progress very rapidly (2-4 years) whilst others can remain disease free for more than 15 years. The explanation for non-progression is not entirely clear but is likely to be multi-factorial in nature.

Chronic immune activation is a hallmark of HIV-1 infection and is already apparent in the acute phase of infection. One of the underlying mechanisms contributing to over-activation of the immune system is microbial translocation, a process whereby bacterial products from the gut enter the circulation due to increased permeability of the epithelial layer. One marker of this phenomenon is the presence of lipopolysaccharide (LPS) in blood. The increased permeability is caused by homeostatic imbalance in the gut with a central role for Th17 cells herein.

1.4 HIV-1 envelope
The HIV-1 env gene encodes for the gp160 kilodalton envelope protein which upon processing generates the gp41 transmembrane region which non-covalently associates with the gp120 molecule. The viral envelope forms a trimer that associates with the viral membrane and protrudes from the virion. One gp120 molecule consists of five variable (V) and five constant (C) regions, corresponding with their diversity observed among patients. Some constant regions approach the variation generally observed in the variable regions, while a few variable regions remain relatively constant. The V1V2 region of gp120 masks the coreceptor binding site and has been strongly linked to viral infectivity and escape from antibody neutralization. The V3 region, together with specific residues around this loop, is the primary determinant for coreceptor usage. Specifically, alterations in the overall V3 charge and changes to the gp120 N-linked glycosylation pattern have been heavily associated with alterations to coreceptor usage. The HIV-1 gp120 molecule is heavily glycosylated with a large number of potential N-linked glycosylation sites (PNGS). The presence of PNGS has been shown to influence correct folding and processing of the molecule and has also consequences for virus infectivity. Glycosylation also affects how the virus interacts with receptors such as the dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) expressed on dendritic cells. Many studies have described how the presence of gp120 PNGS can provide escape from the effects of neutralizing antibodies by forming a ‘glycan shield’ that obscures the neutralizing epitopes on the envelope structure.

1.5 Coreceptor use
CCR5 and CXCR4 are the two most significant coreceptors enabling HIV-1 entry into target cells. Other chemokine receptors have been shown to support viral entry but
their usage *in vivo* is extremely rare. The virus can switch from CCR5 towards CXCR4 usage as either dual-tropic (R5/X4) or solo X4 using viruses\(^{42,43}\). Many hypotheses as to why HIV-1 switches coreceptor exist, such as expansion of the infected cellular repertoire and its association with acceleration of disease progression\(^{44,45}\). Importantly, individuals who do not demonstrate such a switch also develop symptomatic disease. Irrespective of a switch to CXCR4 usage, a host always maintains CCR5 using variants. Preservation of these CCR5 using variants seems to be required for transmission to a new host, since a new infection is almost exclusively established by a CCR5 using variant\(^{46}\). Switching of coreceptor use occurs more frequently in subtypes B and D, while it is less observed for subtype C viruses\(^{47-49}\).

2. **HIV-1 & Cells of the Immune System**

2.1 **HIV-1 infection of immune cells**

HIV-1 infects a variety of cell types including CD4\(^+\) and CD8\(^+\) lymphocytes, monocytes, macrophages, thymocytes and DCs, whereby the CD4\(^+\) lymphocyte population is the predominantly targeted cell type. HIV-1 infects CD8\(^+\) lymphocytes and DCs to low levels and these cells also produce little virus\(^{50-53}\). Infection of multipotent progenitor cells and mast cells has also been described in the literature\(^{54,55}\). Macrophages are infected to a lesser extent than lymphocytes and may produce HIV-1 in a different manner\(^{56-60}\). These cells are less sensitive to (virus induced) apoptosis and are therefore capable of maintaining virus production for an extended period of time.

2.2 **The role of DC-SIGN in virus transmission**

Dendritic cells are involved in transmission of HIV-1 to CD4\(^+\) lymphocytes, which can occur *in cis* and *in trans*. *In cis* transmission occurs when DCs are infected and newly produced virions are transmitted to CD4\(^+\) lymphocytes\(^{40,61}\). Transmission *in trans* takes place via DC-SIGN and/or other C-type lectins that can capture HIV-1 and transmit the virus to CD4\(^+\) T cells\(^{62}\). DC-SIGN interacts with HIV-1 through a number of carbohydrate molecules on the viral gp120 envelope\(^{63}\).

2.3 **Classification of CD4\(^+\) T cell subsets**

The CD4\(^+\) lymphocyte population consists of naïve, memory and effector cells that can be further classified. Effector cells are classified according to their cytokine profile and their function\(^{64}\). T helper 1 (Th1) cells produce high levels of interferon gamma (IFN-γ) and control in part the cell-mediated immune response. They can activate macrophages as well as cytotoxic T lymphocytes (CTLs) and function predominantly against intracellular pathogens. T helper 2 (Th2) cells induce B lymphocytes to produce antibodies for combating extracellular pathogens. To this end, Th2 cells express interleukins such as IL-4, IL-5, IL-9 and IL-13. Regulatory T cells (Tregs) are characterized by expression of TGF-β and FoxP3. They are of importance for maintenance of self-tolerance, suppression of inflammation and prevention of autoimmunity\(^{65,66}\). Th17 cells form a separate population of effector cells that produce IL-17, IL-22 and IL-26. A proportion of these cells can also produce IFN-γ\(^{67}\). They play a role in mucosal immunity by attracting
neutrophil granulocytes, induce expression of antimicrobial peptides and also play a role in autoimmunity and tissue inflammation\textsuperscript{68,69}. Since Th9 and Th22 cells are recently designated as separate T helper subsets, much remains to be elucidated on their role in immunity. Th9 cells may also play a role in tissue inflammation and IL-9 is further involved in differentiation of Th17 cells and Treg function\textsuperscript{70,71}. Th22 cells may play a role in skin homeostasis and pathology\textsuperscript{72,73}.

Memory cells can be subdivided according to their expression profile of chemokine receptors and effector function\textsuperscript{74}. The variant subpopulations are designated as central memory, transitional memory, effector memory and terminally differentiated effector memory cells. Naïve and central memory cells circulate between blood and secondary lymphoid tissues, whilst effector memory cells are preferentially located in non-lymphoid tissues such as lung and gut\textsuperscript{75}. Central memory cells express the memory marker CD45RO and similar to naïve lymphocytes express CCR7 and CD62L. This allows them to extravasate through high endothelial venules (HEV) and migrate to secondary lymphoid organs\textsuperscript{74,76}. When compared with naïve lymphocytes, they have a higher sensitivity to antigenic stimulation upon which they produce mainly IL-2. Upon stimulation they can differentiate into effector memory cells. Central memory cells survive for years and provide long-lasting immunity\textsuperscript{77}.

Effector memory cells do not express CCR7 and are heterogeneous for CD62L expression\textsuperscript{78}. They express a specific set of chemokine receptors and adhesion molecules allowing them to migrate towards inflamed tissue. These cells are short-lived and capable of immediate effector functions. They are more prone to apoptosis than central memory cells, which are more responsive to homeostatic proliferation. Terminally differentiated effector memory cells regain expression of CD45RA which is also present on naïve lymphocytes\textsuperscript{79}.

2.4 The role of CD4\textsuperscript{+} T cell subsets in HIV-1 infection

One aspect of HIV-1 pathogenesis is immune maturation with increased differentiation of naïve and central memory CD4\textsuperscript{+} T cells towards an effector (memory) phenotype\textsuperscript{80,81}. The enormous loss of gut lymphocytes must be restored by production of new memory lymphocytes for maintenance of local immunity\textsuperscript{18,82}. Reduced thymic output and increased differentiation into effector memory cells imposes an increased constraint on naïve and/or central memory CD4\textsuperscript{+} lymphocytes\textsuperscript{17,83,84}. High CCR5 expression renders effector memory cells excellent targets for HIV-1 infection\textsuperscript{85}. The increased differentiation and direct infection of naïve and central memory cells results in increased cellular turnover. Disruption of lymph node architecture by collagen deposition also affects lymphocyte homeostasis\textsuperscript{86,87}. The level of central memory cells decline to such an extent that they are unable to maintain effector memory cell numbers, eventually resulting in overt disease or AIDS\textsuperscript{88,89}.

2.5 Infectivity of CD4\textsuperscript{+} T cell subsets

In primary HIV-1 infection memory CD4\textsuperscript{+} lymphocytes are the predominant infected cell type\textsuperscript{16,20,90}. The exact phenotype of these cells is under debate\textsuperscript{18}, but due to their high cell number in the gut and high CCR5 expression, effector memory cells are likely to
make up the bulk of this population. CCR5 expression levels are low to undetectable on naïve cells and higher on central memory cells. Naïve cells express more CXCR4 than central and effector memory cells. In the chronic phase of infection, the central memory subset is the predominant targeted cell type in most HIV-1 infected patients. Despite low to undetectable CCR5 expression, naïve cells can be infected with CCR5 using variants with a role for the lymphoid tissue environment in providing the necessary stimuli. From *in vitro* studies it is known that CCR5 using variants preferentially infect effector memory cells, while CXCR4 using variants have a preference for naïve cells. *In vivo*, a similar coreceptor usage profile for HIV-1 residing in naïve and memory cells was observed.

Macrophages are infected to a lesser extent than CD4+ lymphocytes and monocytes contain even less virus than macrophages. Differentiation of monocytes into macrophages (MDM) facilitates productive infection of these cells but polarization of MDM into type 1 or type 2 macrophages inhibits HIV-1 replication. HIV-1 produced by macrophages versus lymphocytes differs in phenotypic characteristics that have been shown to influence HIV-1 pathogenesis, such as coreceptor usage, infectivity and sensitivity to antibody neutralization. Macrophages produce virions that are more infectious and that harbor a broader infection profile. Such analyses have not been performed for virus produced by different lymphocyte populations such as Th1 or Th2 CD4+ lymphocytes. Studies into HIV-1 infection of these lymphocyte populations have been limited to the height of infection levels and the profile of virus production. Th2 cells, despite harboring lower CCR5 surface expression levels, have been shown to produce virus quicker and often to higher titers, compared with the Th1 population. Higher intracellular levels of CC-chemokines may also interfere with virus production from Th1 cells.

3. **ANTIRETROVIRAL THERAPY & THE ROLE OF CD4+ T CELL SUBSETS**

Antiretroviral therapy (ART) has proven to be very effective in reducing HIV-1 replication and maintain undetectable viral loads for many years. Antiretroviral therapy can also restore the maturation of the immune response, i.e. restore the balance between the variant CD4+ lymphocyte populations. Therapy may not always result in restoration of central memory function. Although HIV-1 in productively infected lymphocyte subsets decays at similar rates, characterization of the virus population early after start of therapy has not been described. Such analyses may elucidate the sensitivity to therapy of the viral quasispecies in lymphocyte subpopulations.

4. **GENERATION OF HIV-1 PRIMARY ISOLATES**

Isolating HIV-1 from patients is inherently difficult to perform. This is related to the low infectivity of the virus, target cell type, cell density and viral half-life. Other factors involved are expression levels of HIV-1 (co-)receptors and interfering
host factors such as antibodies and CC-chemokines\textsuperscript{121-124}. Major advances have been made in isolating virus from plasma using polybrene\textsuperscript{118}, spinoculation\textsuperscript{125} or using CD44 microbeads\textsuperscript{126,127}. Due to more efficient transmission of cell-associated over cell-free virus\textsuperscript{128-130}, patient primary isolates are usually obtained through coculture with donor peripheral blood mononuclear cells (PBMC)\textsuperscript{131}. Another disadvantage of this method is donor variation in PBMC susceptibility to HIV-1 infection, which may result in a prolonged culture period before virus can be harvested\textsuperscript{132,133}. The method also requires frequent addition of fresh lymphocytes and monitoring by CA-p24 enzyme-linked immunosorbent assay (ELISA) for virus production\textsuperscript{134}. Furthermore, cocultivation with donor PBMC usually results in outgrowth of a specific virus population or populations, such as reactivation of archival strains, which is not a good representation of the circulating strains found in a patient\textsuperscript{135-139}. Apart from sequence differences, primary isolates obtained from PBMC may also differ in sensitivity to neutralization\textsuperscript{140}. Only a few attempts using cell lines for the generation of primary isolates have been described but these studies did not always compare those isolates with the circulating HIV-1 strains in the patient\textsuperscript{141-143}.

5. HIV-1 & CO-INFECTION WITH MYCOBACTERIUM TUBERCULOSIS

Tuberculosis (TB) is one of the most common opportunistic infections observed with HIV-1 infected persons in Sub-Saharan Africa as well as a frequent manifestation of progressing to AIDS\textsuperscript{144} (http://www.who.int/tb/challenges/hiv/factsheet_hivtb_2009.pdf). HIV-1 infection facilitates infection with Mycobacterium tuberculosis (MTB), which increases HIV-1 disease course, plasma viral loads and viral replication in the lung\textsuperscript{145-149}. The underlying mechanism is a difference in eliciting pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and IL-6. In individuals co-infected with HIV-1 and MTB, increased generalized immune activation over HIV-1 positive persons without TB was observed with higher levels of pro-inflammatory cytokines detected, both systemically and at the site of MTB infection\textsuperscript{150-153}. This cytokine milieu enhances HIV-1 replication with a clear role for TNF-α, which induces viral replication through increased activation of NF-κB\textsuperscript{154-156}. Distinct MTB strains can influence viral replication differently, with one strain more profoundly affecting levels of virus replication than another\textsuperscript{157}. A different mechanism of immune interference by MTB infection is induction of IL-10 production by DCs and macrophages upon binding to DC-SIGN\textsuperscript{158}. This suppresses DC activation as well as maturation and may compromise an efficient immune response. Direct cell contact with MTB-specific lymphocytes has been shown to be required for optimal macrophage activation\textsuperscript{159}. This interaction maximizes HIV-1 replication in alveolar macrophages and co-infection of both pathogens in monocytes also increases HIV-1 replication\textsuperscript{160,161}. There is however one contradictory publication which describes inhibition of HIV-1 replication in MTB-infected monocyte-derived macrophages\textsuperscript{162}.

Apart from the effects of MTB on HIV-1 pathogenesis, HIV-1 also influences MTB infection. Besides facilitating infection with MTB, HIV-1 infection also induces development of active TB, which is kept under control in 90% of the MTB-infected
persons without HIV-1\textsuperscript{163-165}. Upon HIV-1 infection, MTB-specific cells are rapidly lost, while cytomegalovirus (CMV)-specific CD4\textsuperscript{+} lymphocytes are still detectable in the late chronic phase of HIV-1 infection\textsuperscript{166,167}. MTB-specific CD4\textsuperscript{+} lymphocytes produce IFN-γ and TNF-α that activate macrophages and contribute to intracellular containment of this pathogen as well as formation of granulomas\textsuperscript{168-171}. Upon HIV-1 infection, MTB-specific lymphocytes produced less IFN-γ \textit{in vitro} compared with HIV-1 uninfected persons\textsuperscript{150}. From these observations it can be concluded that both pathogens can have a negative impact on each other.

6. HIV-1 & OTHER CO-INFECTIONS

Besides MTB, pathogens such as Plasmodium falciparum, the causative agent of malaria, and helminths also pose a health threat to HIV-1 infected individuals. Their high prevalence in Sub-Saharan Africa overlaps with the severe prevalence of HIV-1 infection in this region, suggesting co-infection of HIV-1 with one of these other microbes to be a common event (http://www.who.int/topics/en). Increased immune activation is frequently observed among these individuals, which can influence HIV-1 replication and pathogenesis\textsuperscript{25}.

Malaria can affect HIV-1 infection in various ways and HIV-1 can also be detrimental for malaria disease course and compromise anti-malaria immunity\textsuperscript{172,173}. P. falciparum infection results in an increase in HIV-1 viral load in co-infected individuals, which has been confirmed by observations where anti-malaria treatment can reduce HIV-1 plasma viral loads\textsuperscript{174,175}. The pro-inflammatory response to malaria appeared to be the underlying cause of the enhanced viral replication, since viral load correlated with markers of immune activation\textsuperscript{176,177}. TNF-α plays a role here by influencing HIV-1 transcription by inducing factors that act on the long terminal repeat (LTR). The influence of malaria infection on HIV-1 disease progression or mortality rates is inconclusive, in part due to difficulties in performing such studies\textsuperscript{173,178,179}. However, one study observed an association between malaria infection and a more rapid decline in CD4\textsuperscript{+} T cell counts\textsuperscript{180}. Similar to what was observed in MTB infection, more virus was derived from macrophages\textsuperscript{176}. Also, the malarial pigment hemoglobin promoted HIV-1 transmission by DCs that had a more mature phenotype, possibly explaining for the increased transmission of HIV-1\textsuperscript{181}.

Schistosomiasis is an example of a chronic helminth infection and these infections have been shown to impact HIV-1 pathogenesis by skewing the immune response towards a Th2 phenotype\textsuperscript{182-185}. Although such infections have been associated with increased plasma viral load, parasite clearance does not always result in reduction of this parameter\textsuperscript{186-188}. The conclusion from an overview of three other studies was that helminth treatment delayed HIV-1 disease progression by reducing plasma viral load or increasing CD4\textsuperscript{+} counts\textsuperscript{189}. On another level, Schistosomiasis infection may influence HIV-1 infection by attracting HIV-1 infected mast cells to the gut. These cells have been described as being cellular reservoirs for HIV-1 and re-activation of these cells may induce viral replication\textsuperscript{15,190}. Schistosomal eggs may play a role herein by inducing virus production by these cells through IgE cross-linking\textsuperscript{191}. 


7. Thesis Outline

In this thesis we studied the influence CD4+ cell types have on HIV-1 infection. Virus diversity correlates with viral load and fitness and when studied longitudinally gives insight into what drives disease progression. To gain a better understanding of the role the variant CD4+ lymphocyte subsets can play in HIV-1 infection we extensively analyzed the genetic make-up of the viral quasispecies residing in naïve, central memory as well as effector memory CD4+ lymphocyte subsets and quantified HIV-1 infection levels (Chapter 2). We longitudinally studied the events that occurred in an individual who switched in coreceptor usage versus one who did not. We observed differences in infection levels among the studied CD4+ lymphocyte subsets, but this had no influence on the viral quasispecies within these subsets, also not in the individual who switched coreceptor usage. In Chapter 3 we followed up on this study to assess the influence of antiretroviral therapy on the viral quasispecies in the variant CD4+ lymphocyte subpopulations. Two to five weeks after therapy initiation we observed no effect on HIV-1 residing in naïve and central memory CD4+ lymphocytes.

Chapter 4 describes a cell-line based method we developed to generate HIV-1 primary isolates. By cocultivation of patient PBMC with U87.CD4 cells expressing the CCR5 coreceptor, we generated primary isolates in 86% of individuals with a viral load higher than 7,000 copies per ml. Virus production was monitored by syncytia formation without requirement for CA-p24 ELISA. We sequenced the obtained primary isolates and compared them with circulating strains from the patients and also assessed their infectivity on multiple cell types. We performed this assay in Ghana where we also included patients for a study on the influence of MTB on HIV-1 infection (Chapter 5). From identified co-infected individuals we quantified cells specific for MTB and CMV. The phenotype of these pathogen-specific CD4+ T cells were characterized in detail to shed light on possible mechanisms that may explain for the disparity in decline of CD4+ T cells among persons harboring different co-infections. We further characterized specific CD4+ T cell subpopulations of MTB-responsive cells that were most infected by HIV-1. In the final chapter of this thesis, we investigated whether a specific producer cell type can influence the phenotype of the generated HIV-1 virions (Chapter 6). We generated and compared HIV-1 virus stocks produced on Th1 versus Th2 lymphocytes and macrophages versus CD4-enriched lymphocytes. We assessed their sensitivity to coreceptor blocking agents and 2G12 antibody neutralization, and their ability to be transmitted by cells expressing DC-SIGN to CD4+ lymphocytes. All findings from this thesis are described in Chapter 7 and discussed in the context of current literature, specifically with regards to the implications for HIV-1 pathogenesis.
Chapter 1

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Chapter 1


