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INTERDISCIPLINARY CHARACTERIZATION OF T CELL DYNAMICS IN HIV INFECTION

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To my family

ABSTRACT

HIV has caused one of the most devastating pandemics in modern medicine. HIV infects and kills on of the central players in effector immunity, CD4+ T cells, that provide helper mechanisms to all arms of the immune system. Although the virus indirectly affects most cells of the immune system, CD4+ and CD8+ T cells in particular become highly dysfunctional and show traits of severe immune pathology during the infection. These cells are of importance in adaptive immunity and recognize through their T cell receptors foreign antigens that are presented on MHC molecules. In the absence of normal T cell dynamics and homeostasis, host effector immunity collapses and most individuals develop AIDS, without antiretroviral therapy.

The growing number of immunological variables measured today poses challenges to studying T cell dynamics in HIV infection. However, with the introduction of new techniques within bioinformatics, we now possess statistical tools to analyse combined measurements of T cell pathology, epitope targeting and dysfunction in the context of HIV infection. In all of these studies, multi-parametric flow cytometry and advanced bioinformatics were thus combined to study traits of T cell dynamics in HIV infection. By examining a broad range of T cell markers, we concluded in **paper I** that the CD4/CD8 ratio correlated with a significantly increased number of pathological T cell populations and was associated with CD4 recovery 2 years after ART initiation. These data indicate that the CD4/CD8 ratio would be a suitable clinical predictor of combined T cell pathology in HIV infection.

By developing a novel epitope selection algorithm in **paper II**, we aimed to identify optimal MHC class II-restricted HIV epitopes with broad viral and host coverage. Employing both immunological and virological approaches, a set of peptides was shown to induce broad HIV-specific CD4+ T cell responses, where the number of targeted Gag epitopes was inversely correlated with HIV viral load. In order to further trace events of HIV disease progression, we investigated whether the combined pattern of HIV evolution and CD8+ T cell functionality could explain the risk of HIV disease progression in HLA-B*5701+ patients (**paper III**). HIV Gag sequence diversity was shown to be lower and multi-functional responses higher against wild-type and autologous HLA-B*5701-restricted epitopes in subjects of low risk of disease progression. Both of these studies highlight the power of multidisciplinary approaches, integrating complex evolutionary and immunological data, to understand the mechanisms underlying T cell dysfunction and pathogenesis.

To further clarify why HIV-specific CD8+ T cells exhibit severe dysfunctional characteristics in both treated and untreated HIV infection, we studied in **paper IV** the role of two central T-box transcription factors (T-bet and Eomes) using combined flow cytometry and bioinformatics. It was shown that HIV-specific CD8+ T cells almost exclusively have highly elevated levels of Eomes, but lower T-bet expression, which is associated with up-regulation of numerous inhibitory receptors, impaired functional characteristics and a transitional memory differentiation status. Surprisingly, these features were retrained despite many years on ART, implicating that the relationship between T-bet and Eomes might partly explain the inability of CD8+ T cells to control viral rebound post ART cessation.

In summary, this thesis has combined the knowledge of immunology and virology with the help of bioinformatics to study T cell dynamics in HIV infection. This interdisciplinary approach has increased our knowledge of factors that are linked to T cell pathology, risk of disease progression and impaired T cell functionality.

LIST OF PUBLICATIONS

I. **Marcus Buggert**, Juliet Frederiksen, Kajsa Noyan, Jenny Svärd, Babilonia Barqasho, Anders Sönnerborg, Ole Lund, Piotr Nowak, Annika C. Karlsson. *Multiparametric Bioinformatics Distinguish the CD4/CD8 Ratio as a Suitable Laboratory Predictor of Combined T Cell Pathogenesis in HIV Infection*. **J Immunol.** 2014, 192. Feb 3 [Epub ahead of print].

II. **Marcus Buggert**, Melissa Norström, Chris Czarnecki, Emmanuel Tupin, Ma Luo, Katarina Gyllensten, Anders Sönnerborg, Claus Lundegaard, Ole Lund, Morten Nielsen, Annika C Karlsson. *Characterization of HIV-Specific CD4+ T Cell Responses against Peptides Selected with Broad Population and Pathogen Coverage.* **PLoS One**. 2012;7(7):e39874.

III. Melissa M Norström, **Marcus Buggert**, Johanna Tauriainen, Wendy Hartogensis, Mattia C Prosperi, Mark A Wallet, Frederick Hecht, Marco Salemi, Annika C Karlsson. *Combination of immune and viral factors distinguish low-risk versus high-risk HIV-1 disease progression in HLA-B*5701 subjects.* **J Virol**. 2012 Sep;86(18):9802-16.

IV. **Marcus Buggert**, Johanna Tauriainen, Takuya Yamamoto, Juliet Frederiksen, Martin Ivarsson, Jacob Michaelsson, Ole Lund, Bo Hejdeman, Marianne Jansson, Anders Sönnerborg, Richard A. Koup, Michael R. Betts, Annika C. Karlsson. *T-bet and Eomes are differentially linked to the exhausted phenotype of CD8+ T cells in HIV infection*. **Manuscript**.

RELATED PUBLICATIONS NOT INCLUDED

I. Ilka Hoof, Carina L Perez, **Marcus Buggert**, Rasmus KL Gustafsson, Morten Nielsen, Ole Lund and Annika C Karlsson. *Interdisciplinary Analysis of HIV-specific CD8+ T Cell Responses Against Variant Epitopes Reveals Restricted T Cell Receptor Promiscuity.* **J Immunol**. 2010 May 1;184(9):5383-91

II. Carina L Perez*, Jeffrey M Milush*, **Marcus Buggert**, Emily M Eriksson, Mette V Larsen, Teri Liegler, Wendy Hartogensis, Peter Bacchetti, Ole Lund, Frederick M Hecht, Douglas F Nixon, Annika C Karlsson. *Targeting of conserved Gag epitopes in early HIV infection is associated with lower plasma viral load and slower CD4+ T cell depletion.* **AIDS Res Hum Retroviruses**. 2013 Mar;29(3):602-12

III. **Marcus Buggert**, Melissa M Norström, Frederick Hecht, Marco Salemi, Annika C Karlsson. *Functional avidity and IL-2/perforin production is linked to the emergence of mutations within HLA-B*5701-restricted epitopes and HIV-1 disease progression*. **Manuscript**, major revision, J Immunol. 2014.

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LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
APOBEC	Apolipoprotein B messenger RNA editing enzyme catalytic polypeptide-like
ART	Antiretroviral therapy
ARV	Antiretroviral
bNAb	Broadly neutralizing antibodies
CD	Cluster of differentiation
CMV	Cytomegalovirus
CPE	Cytopathic effect
CRF	Circulating recombinant form
DC	Dendritic cell
DNA	Deoxyribonucleic acid
ELISPOT	Enzyme-linked immunospot
Env	Envelope
Eomes	Eomesodermin
ER	Endoplasmatic reticulum
FACS	Fluorescent activating cell sorting
Gag	Group-specific antigen
HAART	Highly active antiretroviral therapy
HIV	The human immunodeficiency virus type 1
HLA	Human leukocyte antigen
HPC	Hematopoietic stem cells
HRP	High risk progressor
IDU	Intravenous drug users
Ig	Immunoglobulin
IN	Integrase
LCMV	Lymphocytic choriomeningitis virus
LTNP	Long-term non-progressor
LTR	Long terminal repeat
LRP	Low risk progressor
MHC	Major histocompatibility complex
Nef	Negative regulatory factor

NK	Natural killer
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleoside reverse transcriptase inhibitors
PBMC	Peripheral blood mononuclear cell
PCA	Principal component analysis
PCR	Polymerase chain reaction
Pol	Polymerase
PR	Protease
Rev	Regulator of virion gene
RNA	Ribonucleic acid
RT	Reverse transcriptase
SIV	Simian immunodeficiency virus
SGS	Single genome sequencing
SPICE	Simplified presentation of incredibly complex evaluations
TCR	T cell receptor
Tat	Transcriptional transactivator
Tfh	Follicular T helper cells
Th	T helper
Vif	Virion infectivity
Vpr	Viral protein R
Vpr	Viral protein R

1. INTRODUCTION

1.1 THE HUMAN IMMUNODEFICIENCY VIRUS

1.1.1. The discovery of HIV

The human immunodeficiency virus type 1 (HIV)* became apparent to the world almost a century after the first human was infected with the virus. In 1981 the Centre for Disease Control wrote a brief article about 5 previously healthy homosexual men who had developed *Pneumocystis* pneumonia, possibly due to severe cellular immune dysfunction (*1*). Soon after, other case reports of rare opportunistic diseases in different parts of the United States were documented, particularly in homosexual men (*2, 3*), but also intravenous drug users (IDUs), hemophiliacs and immigrants from Haiti (*4, 5*). It became apparent to the scientific community that something was deteriorating the cellular immune system in these individuals and the disease was therefore named acquired immunodeficiency syndrome, or AIDS (*6*). In 1983, the French scientists Françoise Barré-Sinoussi and Luc Montagnier isolated a T-lymphotropic retrovirus from the secondary lymph nodes of an AIDS patient (*7*), this virus was later named HIV. The French scientists later shared the Nobel Prize for their discovery of the virus. Shortly after HIV was discovered, Luc Montagnier and colleagues also discovered a second ancestor of the virus, named HIV-2 (*8*). However, HIV-2 is not as aggressive as the type 1 virus, which accounts for the vast majority of deaths in the HIV/AIDS pandemic we know of today.

* The term "HIV" will be used to refer to the human immunodeficiency virus type 1 in this thesis unless otherwise stated.

1.1.2 The origin of HIV

The origin of HIV and how it crossed the species barrier has been a subject of great discussion for many years in the society. Today we know that HIV originally emerged through several cross-species transmissions of the simian immunodeficiency virus (SIV) in non-human primates to humans (*9, 10*). Through various phylogenetic analyses, a recent study found that non-human primates were infected with SIV at least 32,000 years ago, but the virus might be even older (*11*). It is hard to determine exactly when HIV was shaped and crossed the species barrier between non-human primates and humans, but statistical models have verified that it took place about 100 years ago (*12*). It is thought that SIV was originally transmitted from chimpanzees (*Pan troglodytes troglodytes*) to humans in Western Africa generating HIV (*9*). The transmission creating HIV-2, on the other hand, took place in the same region in 1940, from sooty mangabeys (*Cercocebus atys atys)* (*13*). Why the transmission happened in the beginning of the 20th century remains unknown. A common explanation of how the virus transmitted and then spread globally stems from the social behavior of people in these regions due to colonization and wars that forced people to migrate and urbanize (*14*). For some time people thought the transmission between the species as due to contamination from chimpanzee tissues in the preparation of the oral polio vaccine (*15*). However, the SIV strains in these parts of Africa (Democratic Republic of Congo), from which the virus would have been contaminated, are not phylogenetically

related to the HIV strains circulating in the world today, therefore providing evidence that these chimpanzees did not transmit the virus to humans (*16*).

1.1.3. HIV genome and structure

HIV is a lentivirus and belongs to the family of retroviruses (*Retroviridae*). Lenti means “slow” in Latin and refers to the slow disease progression that is caused by these viruses. While other retroviruses have been found to infect humans (*e.g*. HTLV-1), no other lentivirus has been detected in humans. HIV is like all retroviruses: an enveloped virus containing two copies of positive single-stranded RNA. As is typical for all lentiviruses, HIV also contains a canonical capsid surrounding the genetic material, surrounded by a matrix to stabilize the viral core. Within the capsid, three viral enzymes are located that are important for the replication cycle: reverse transcriptase (RT), integrase (IN) and protease (PR). The envelope is composed of a lipid bilayer derived from human cells. All of these molecules create the core of the HIV particle, distinguishable in **Figure 1**.

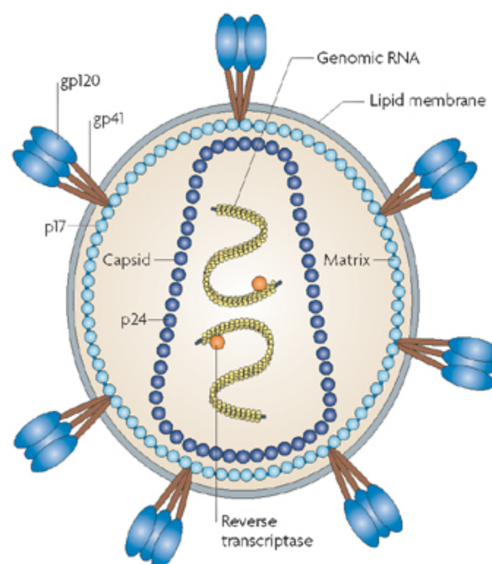


Figure 1. Structure of the HIV virion. Reprinted with permission from (*184*).

The HIV genome consists of about 10,000 base pairs, where the genome is composed of nine open reading frames. Three of the open reading frames are found in all retroviruses: the group-specific antigen (*gag*), envelope (*env*) and polymerase (*pol*), encoding the structural proteins. HIV *gag* originally encodes a polyprotein (p55), which is cleaved by protease into the capsid (p24), matrix (p17), nucleocapsid (p7) and p6. HIV *env* is transcribed into another polyprotein (gp160), and subsequently spliced by the protease into gp120 (surface protein) and gp41 (transmembrane protein) creating the viral spikes. HIV *pol* encodes the viral enzymes RT, INT and PR. HIV also consists of two regulatory proteins: transcriptional transactivator (Tat) and regulator of virion gene expression (Rev). Both of these proteins are expressed early in the viral life cycle and important for viral gene expression. In addition, HIV encodes four regulatory proteins: negative regulatory factor (Nef), viral protein r (Vpr), viral protein u (Vpu) and viral infectivity factor (Vif). These proteins are primarily important for immune evasion/pathological functions (*17*).

1.1.4. HIV replication

HIV replicates rapidly and studies have shown that a human cell infected with HIV might produce over 10^4 virions during its life span. The virus infects a host cell by binding through its viral spikes to the target molecule cluster of differentiation (CD) 4. Using its surface protein gp120, HIV binds to CD4, enabling a conformational change and docking to specific co-receptors (CCR5 and CXCR4). This brings the virus close enough for the transmembrane protein gp41 to penetrate the membrane and facilitate the fusion of the virus with the cellular membrane and release of the viral material into the cell. RT then reversely transcribes only one of the single copies of HIV ribonucleic acid (RNA) into a double stranded deoxyribonucleic acid (DNA) molecule that is transported into the nucleus as a pre-integration complex. The complex is then integrated through catalyzation by the IN and remains stable as a provirus until transcriptional processes activate the virus again. The provirus can remain stable for many years in resting cells due to the integration into the human genome, and is thought to be the reason for viral latency. The genome of HIV possesses long term repeats (LTR) at the ends of the genome, where human Polymerase II binds and starts the transcription upon cellular activation. At first, the HIV proteins Nef, Tat and Rev are produced, where Tat binds to the transactivation response region (TAR) downstream of LTR and facilitates further elongation of mRNA by Polymerase II. Rev then docks to the rev responsive element (RRE) on *env* to facilitate the exportation of the mRNA from the nucleus and into the cytoplasm. Through the normal machinery of cellular transcription, the viral mRNA is subsequently translated near the endoplasmatic reticulum (ER). Spliced and un-spliced variants of the viral proteins are transported through the Golgi and ER complexes to the cellular membrane, where the particles assemble. The viral particle is released by budding from the cellular membrane and finally matures when the gag-pol polyprotein is cleaved by PR to create the functional proteins (*18*) (**Figure 2**).

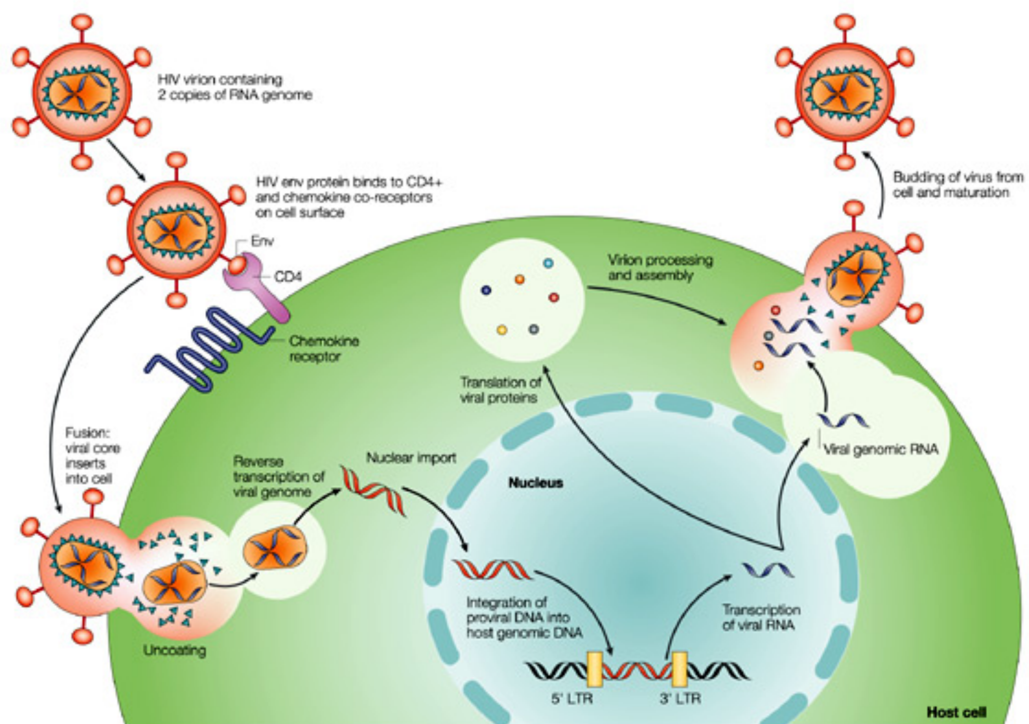


Figure 2. HIV replication cycle. Reprinted with permission from (*19*).

1.1.5. Genetic diversity

HIV possesses a very high replication capacity and due to the huge number of cells (10^8) that are productively infected with the virus, over $10^{9\text{-}10}$ viral particles are produced every day (*20, 21*). This high replication rate, together with the ability of the virus to mutate, have contributed to the inability to develop effective vaccines that are able to clear or protect us against the virus. The remarkable diversity of HIV is due to several different characteristics that retroviruses like HIV possess. First of all, the HIV enzyme RT is error-prone, leading to point mutations during the reverse transcription of RNA to DNA. This is the leading cause of the extensive genetic diversity of HIV. In total, 0.1-0.3 mutations occur per genome for every replication cycle (*22*) and, due to lack of cellular proof-reading mechanisms, these mutations will not be corrected as in normal cells. Because of the extensive replication rate of HIV, point mutations can occur up to 10^4 times per day. In addition, the RT switches between the two single-stranded RNA molecules during the reverse transcription process, which creates hybrids (recombinations) of the DNA molecules (*23*). Many of the circulating HIV subtypes in the world today exist because of recombination. Like RT, the RNA Polymerase II also lacks proof-reading mechanisms during the conversion of viral DNA to mRNA, and is also a source of point mutations. Nevertheless, the pressure from the human immune system and host-restriction factors like apolipoprotein B messenger RNA editing enzyme catalytic polypeptide-like (APOBEC) means that the virus further diversifies and evolves to escape the host (*24*).

For the reasons listed above, HIV has evolved since its introduction in the early 20th century to humans. HIV is divided into three groups: M (Main), O (Outliers), and N (Non M or O). Recently, a new group of HIV called P was discovered, which is more related to the SIV strains seen in gorillas (*25*). However, the only group that is globally spread is M, which can be further divided into the different subtypes A, B, C, D, F, G, H, J, K and almost 50 circulating recombinant forms (CRFs) (www.hiv.lanl.gov). While subtype B is the most pronounced in the Western World (Europe and North America), most HIV patients in the world are infected with subtype C, primarily found in Sub-Saharan Africa. Around 90% of HIV infected people globally are infected with either A, B, C, D and two CRFs (CRF01_AE and CRF02_AG) (*26*). At the beginning of the pandemic, when HIV was discovered, most of the infected individuals in Sweden were transmitted with subtype B. However, due to the increasing number of individuals becoming infected with CRFs, subtype CRF01_AE has been modeled to become more prevalent in 2015 than subtype B (Personal communication, Anders Sönnerborg).

In order to study the genetic diversity and evolution of HIV at the molecular level, methods like phylogenetic analysis have been developed. This method allows researchers to determine the relationship between different sub-species of HIV through molecular sequencing. Phylogenetic analyses are based on mathematical models, or algorithms, that generate tree structures to relate different sequences to one another. Using phylogenetic analysis, we now know when and where HIV originally originated and thereafter spread to the rest of the world (reviewed in (*27*)).

1.1.6. HIV today

Although the introduction of anti-retroviral therapy (ART) has changed the impact of the morbidity rates of AIDS, the spread of HIV worldwide is substantial. Since the beginning of the pandemic, HIV has spread to over 75 million people, of whom approximately half have died from AIDS. Today, more than 35 million people are infected with HIV worldwide and 1.6 million die every year due to AIDS. Importantly, 2.3 million people become infected every year, leading to an increase of HIV prevalence across the globe (*28*). To some extent this is good news, as it means that fewer people are dying because of AIDS. However, it also means that the costs for society will increase due to increasing supplementation of ART. This is one of the major reasons why it remains important to develop an effective vaccine, or another therapeutic approach, that would be able to either protect against or eradicate the virus in the future.

1.2 MANAGEMENT OF HIV

1.2.1 Antiretroviral therapy (ART)

In the mid-1990s, several studies were published showing that combinations of 3 different antiretroviral (ARV) drugs drastically reduced the HIV RNA copies in blood, decreased the risk of AIDS morbidity and decelerated HIV disease progression compared to single/double drug regimens (*29, 30*). The combination of three different ARVs was soon incorporated in most Western clinics and became the starting point for the highly active antiretroviral therapy (HAART), or combination antiretroviral therapy (ART), era. Although the ART era already began in the mid-1990s, "only" 9.7 million people had access to the combination therapy at the end of 2012 (http://www.who.int/hiv/en/). A total of 15 million people were in acute need of ART at the end of 2011.

Today, five different classes of ARV drugs exist:

- **Entry inhibitors**
  These drugs block the binding of the virus to surface receptors on host cells to inhibit the entry of HIV. Maraviroc, for example, is an allosteric modulator of the human co-receptor CCR5 and thereby prevents binding of gp120 to the receptor (*31*). Some controversy exists as to whether maraviroc might increase or decrease the state of immune activation after intensification with the drug (*32*).

- **Fusion inhibitors**
  This type of drug inhibits the fusion of the virus with the cellular membrane. The only available today within this class is the peptide enfuvirtide, which binds to the HIV gp41 protein and thereby prevents the fusion of the virus to the host cell (*33*).

- **Integrase inhibitors**
  This is a fairly new class of drugs that has been implemented more and more in the clinics. As the name describes, these drugs act by inhibiting the HIV enzyme integrase and thereby preventing the integration of viral DNA into the human genome. Currently, there are several integrase inhibitors available for use in the clinics. The first integrase inhibitor to become approved was raltegravir. This drug acts by competing with Mg^{2+} ions at the metal binding site of the enzyme (*34*). Raltegravir intensification has been proven to increase 2-LTR circles (*35, 36*), and lower immune activation (*36*), implicating that the drug has effects on *de novo* production of HIV particles despite successful combination ART.

- **Protease inhibitors**
  The combination ART era began with the introduction of the protease inhibitors in combination with reverse transcriptase inhibitors (see below). These inhibitors interfere with the HIV enzyme protease and prohibit the final maturation of viral particles by blocking the gag-pol polyprotein cleavage. The first drugs to enter clinics were saquinavir and ritonavir. These drugs, in combination with others, made the number of AIDS related morbidities in the United States drop by over 60% in the mid-1990s. A potential problem with protease inhibitors has been the high degree of resistance against the first generation of drugs. However, new protease inhibitors have been successful against the resistant strains of HIV (*37*).

- **Reverse transcriptase inhibitors**
  -Nucleoside reverse transcriptase inhibitors (NRTIs): This sub-class of drugs blocks reverse transcriptase by acting as nucleoside analogues. The first five ARV drugs (including zidovudine and abacavir) were NRTIs and are today almost always one component in combination ART. NRTIs are competitive substrate inhibitors, meaning that they terminate the RT process by incorporating themselves into a viral DNA chain and through its lack of 3' OH groups prevent other nucleosides from being incorporated (*38*).

  -Non-nucleoside reverse transcriptase inhibitors (NNRTIs): The other subclass of RT inhibitors, also called non-competitive inhibitors of RT. NNRTIs bind near the active site of RT and thereby inhibit the catalyzation of RNA into DNA. NNRTIs, like efavirenz and nevirapine, are often chosen as a first class regimen for combination ART today (*38*).

1.2.2 Routine laboratory parameters

Different factors in the blood are measured in order to monitor the disease progression of HIV. In the Western part of the world, CD4 count (cells/uL), CD8 count (cells/uL), CD4%, CD8%, CD4/CD8 ratio and HIV RNA copies per mL (viral load) are all measurable. However, the most commonly used monitors of health are CD4 count and viral load. While CD4 count is primarily measured to determine the health of a subject and when ART should be introduced, the viral load is monitored to detect whether subjects experience rebound of HIV due to ART resistance and/or poor treatment adherence (*39-41*).

Despite its central role in the clinics today, the absolute number of CD4+ T cells does not always serve as an appropriate surrogate of the state of disease progression and health. Taylor *et al* determined as early as 1989 that the CD4% and CD4/CD8 ratio are slightly better predictors of the rate of progress between time of infection to AIDS development (*42*). Furthermore, while it has long been appreciable that the CD4 count might recover to the "normal" levels of healthy individuals, the CD4/CD8 ratio is rarely fully restored. The inverse relationship between the CD4/CD8 ratio in both adults and adolescents has been linked to the persistent immune activation and exhaustion distinguished in many patients despite long-term ART (*43, 44*). These studies implicate that although the CD4 count might be a fair prognostic factor of disease progression in HIV infection, other monitored laboratory parameters might serve as improved markers for the immunopathological outcomes before and after ART (**paper 1**).

In the wake of studies showing a clear benefit of ART in reducing numbers of transmissions, possibly all affected individuals will be administered with ART in the near future. However, because of socio-economical reasons and other aspects, this seems to be a more distant prospect for developing countries, and therefore routine laboratory parameters will potentially serve as vital information for a long time ahead (*45*).

1.3 THE IMMUNOPATHOGENESIS CAUSED BY HIV

1.3.1 Transmission

HIV is a blood borne disease that is spread between humans through contact with bodily fluids, including blood, vaginal fluids, semen and breast milk. In a vast majority of cases (70-80%), however, HIV is transmitted through sexual contact (*28*). Although at the beginning of the pandemic homosexual intercourse was identified as a main factor in the spread of HIV, heterosexual encounters account for the majority of new HIV infections worldwide (*28*). Infection through blood, on the other hand, is nowadays mostly seen in IDUs through shared needles, while fewer mother-to-child transmissions are identified than previously due to effective administration of preventive ART to the mother and child (http://www.who.int/hiv/topics/mtct/en/).

The probability of heterosexual transmission after a single sexual encounter ranges between 0.01-0.23% (*46*). This wide range is caused by diverse events, like the number of viral copies, which tends to be higher during primary HIV infection and later during the phase of AIDS (*47*). Co-infections, especially sexually transmitted diseases, increase the number of target cells for HIV, and the number of sexual partners (sexual behavior) also increases the risk of HIV transmission. However, the introduction of ART does not only impede the deterioration of immunity following the infection, it also reduces the rate of new HIV infections dramatically by 96% (*48*). This discovery was dubbed the greatest scientific breakthrough of the year in 2011 in Science magazine (*49*). Hopefully, this will aid policy makers in the future to initiate therapy early on for individuals – not only to increase the chances of proper immune reconstitution, but also to decrease the number of new infections.

1.3.2 Cellular tropism

As previously described, HIV docks through its viral spikes to the CD4 molecule. Therefore, the so-called T helper cells, or CD4+ T cells, are those that primarily become infected and thereby depleted by the virus. Due to co-receptor engagement of HIV, the virus productively infects particularly CCR5 and CXCR4 expressing cells (*50, 51*).

Typically, a single (*52*), or multiple (*53*), viral strain(s) disseminating the host first uses CCR5 to infect target cells. CCR5 belongs to the beta chemokine receptor family and binds the chemokine ligands CCL3 (MIP-Iα), CCL4 (MIP-Iβ) and CCL5 (RANTES). These ligands usually create a chemotactic gradient for immune cells to migrate to a peripheral site of infection. The CCR5 receptor is primarily expressed on memory T cells, but also macrophages, dendritic cells (DCs) and microglia (*54*). It is therefore mainly memory CD4+ T cells that are infected through mucosal transmission, but to a minor degree also DCs and macrophages (*55*). Most studies have shown that the first viruses infecting humans are lymphotropic viruses (*56, 57*), meaning that they primarily infect CD4+ T cells. However, depending on the route of transmission, intraepithelial DCs might "catch" HIV through its dendrites (*58*), which then spread the virus to other neighboring cells (*59*). Whether DCs and macrophages are productively infected or capture the virions through cell surface receptors like DC-sign (*60*), remains controversial, as these cells usually express CD4 at much lower frequencies than CD4+ T cells. Still, some evidence implicates that DCs might capture HIV and transfer the virus to secondary lymph nodes (*61*). Usually, when the virus has established itself in the lymph nodes, it rapidly replicates due to a higher abundance of target cells and then disseminates to the rest of the body.

When the virus evolves as a consequence or cause of disease progression, HIV might also infect cells through CXCR4 interaction. This receptor binds to the SDF-1 ligand (*62*) and is important for the homing of hematopoietic stem cells (HSCs) to the bone marrow. Recently, it was therefore implicated that HIV might infect HSCs through CXCR4 engagement and establish infection in the bone marrow (*63, 64*). However, this finding was not confirmed in two recent studies, which showed no evidence of HIV establishment in sorted HSCs from patients (*65, 66*); meaning that further studies must be undertaken to determine whether HSCs might be a reservoir of HIV. In addition to HSCs, most peripheral CD4+ T cells express CXCR4 and these are usually targeted in the later phase of the infection.

1.3.3 The progression to AIDS

The course from an HIV infection to the development of AIDS is characterized by three different phases: acute infection, clinical latency and the AIDS phase of infection. During the first 1-2 weeks after infection, HIV establishes itself at the site of infection and local lymph nodes – if the transmission occurs at mucosal sites. The virus is not measurable in the plasma during these days (eclipse phase) and no symptoms are usually distinguishable. After the virus has established itself in local lymph nodes, it starts to replicate vigorously and disseminate to the rest of the body. This is followed

by a steep viral peak in the plasma, at which point flu-like symptoms usually start to develop . As a consequence, the adaptive immune system will “kick in” and deplete virus-infected cells and neutralize HIV directly. The HIV-specific response together with fewer target cells for HIV leads to a 100-fold lowering of the viremia to a viral set-point. These initial events of the infection sum up the acute phase of the infection, which is divided into specific Fiebig states based on detection of various markers of HIV in the circulation (**paper 3**; **Figure 3**).

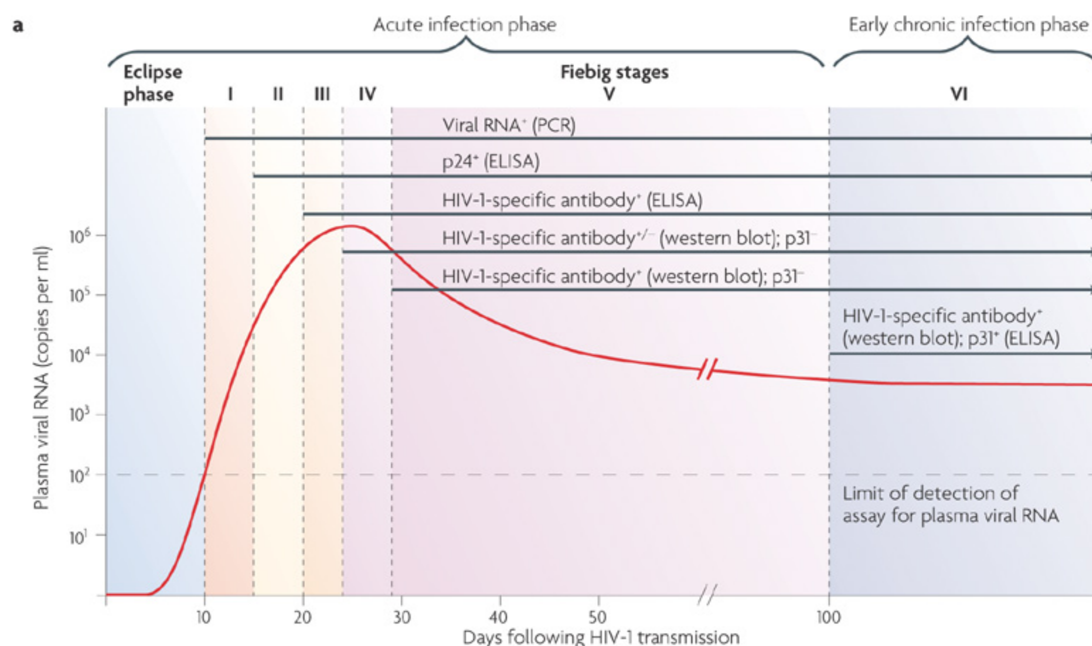


Figure 3. Early events following HIV infection. Reprinted with permission from (*67*).

The clinical latency phase of HIV infection is generally asymptomatic. Despite this, HIV continues to replicate and evolve. There is a constant struggle by the immune system to kill HIV, replenish the loss of CD4+ T cells and balance inflammation. During this phase there might be fluctuating levels of HIV RNA and CD4 counts, but progressively there is most usually a decline in circulating CD4+ T cells. Only a small fraction of those obtaining HIV does not progress towards AIDS: these individuals are usually called long-term non-progressors (See “Correlates of disease progression”).

When the CD4+ T cells have declined considerably, typically to <200 cells/uL or CD4 percentage <14%, AIDS emerges. Without any treatment, the person will usually die after three years due to vulnerability to infections and infection-related cancers (opportunistic infections) (*68*).

1.3.4 Deterioration of immunity

Although it might be reasonable to assume that CD4+ T cells are solely depleted as a consequence of slow cytopathic effects (CPEs) directly executed by HIV, the deterioration of the immune system is more complex than that. The leading reason for this statement is that HIV infects only a small proportion of the CD4+ T cells (1:100-1000) and yet causes the severe deterioration of effector immunity (*69*). Following acute HIV infection, a massive depletion of effector CD4+ T cells occurs at different mucosal sites of the body: *e.g.* the gut, reproduction sites and respiratory airways (*70-*

72). The CD4+ T cells that are primarily lost at these sites are those expressing the co-receptor CCR5, demonstrating that CD4+ T cell depletion is not slow, but rather profound, early after the infection. CD4+CCR5+ T cells at secondary lymphoid tissues are also depleted, but naïve and CCR7+ (lymph-node homing) CD4+ T cells, also called central memory CD4+ T cells, are largely spared from the acute CD4 depletion. Soon after the initial depletion of CD4+ T cells, a rapid proliferation happens to replenish the initial loss of effector (CCR5+) CD4+ T cells. Throughout the chronic phase of infection, central memory CD4+ T cells mobilize and try to proliferate in order to regenerate new effector CD4+ T cells. In the end, however, the body cannot continue or maintain a normal homeostasis and the replenishment of new CD4+ T cells fails, leading to AIDS. The chronic loss of CD4+ T cells is therefore not primarily linked to CPEs of CD4+ T cells, because otherwise AIDS would develop relatively early after the infection (*73*). Instead, other effects that are causes, or potentially consequences, of homeostatic failure must exist.

1.3.4.1 Immune activation and inflammation

Already in 1989, Girogi *et al* published a seminal study showing that CD38 and human leukocyte antigen (HLA)-DR are highly up-regulated during HIV infection (*74*). These two markers represent the golden standard of measurements of immune (T cell) activation today. CD38 is a protein that is expressed on the surface of many immune cells and catalyzes the hydrolysis of cyclic ADP-ribose. CD38 becomes highly expressed upon cell activation due to Ca^{2+} mobilization inside the cell (*75*). HLA-DR is a major histocompatibility complex (MHC) class-II surface receptor that is usually expressed only on antigen presenting cells (APCs) to activate CD4+ T cell responses (see section “activation of adaptive immunity”). However, during cell activation, HLA-DR becomes widely expressed on the cell surface, potentially after increased cell cycle progression and turnover *in vivo (76)*.

The role of immune activation in the process of CD4+ T cell destruction is complex. Potentially, although not entirely proven yet, a high T cell turnover is caused by the elevated immune activation during HIV infection, leading to a short-lived pool of central memory CD4+ T cells and poor homeostasis/regeneration of new effector CD4+ T cells (*73*). The likely involvement of immune activation in the process of CD4 depletion is supported by other studies, showing that T cell activation is a better predictor of HIV disease progression than HIV RNA levels themselves (*77-79*). In addition, increased immune activation has also been shown to be a good predictor of poor CD4 recovery post ART initiation (*80, 81*), further supporting the negative influence of T cell activation on CD4+ T cell regeneration. However, despite that high immune activation of CD8+ T cells also occurs, these cells do not decline as a consequence of HIV infection. Thus, there are very likely other factors that lead to the deterioration of CD4+ T cells.

An increase in several pro-inflammatory factors is distinguishable in chronic HIV infection (reviewed in (*82*)). Particularly, IL-6 and TNF have been found at elevated levels and might be consequences of HIV or cytomegalovirus (CMV) replication, co-pathogens, thymic dysfunction and other variables (*83*). Interestingly, two recent

studies showed that 95 % of all CD4+ T cells are depleted through abortive infection (*84*), in a process called pyroptosis. In contrast to normal apoptosis using caspase-3 mediated pathways, pyroptosis happens through caspase-1, which activates the production of several pro-inflammatory cytokines (including IL-1β). This creates a vicious circle where the inflammation recruits new CD4+ T cells that become abortively infected and release more inflammatory markers until cells become essentially exhausted. Caspase-3 was shown to be present only in those cells that were productively infected with HIV, and accounted for just a small amount (5 %) of the general CD4+ T cell destruction (*85*). Future studies need to clarify *in vivo* whether pyroptosis and immune activation are two sides of the same coin, or if activated cells only have increased caspase-3 activity.

1.3.4.2 Immune exhaustion

The process of immune exhaustion has been studied extensively in the context of chronic viral-specific immunity. However, markers of immune exhaustion have also been found to be elevated in HIV infection, where programmed death 1 (PD-1) has been studied in terms of HIV pathogenesis (*86-88*). PD-1 is an inhibitory receptor that particularly negatively regulates T cell responses upon activation (*89*). Previous studies have shown a close association between the expression of PD-1 and CD38+HLA-DR+ T cells in HIV infection (*90*). In addition, PD-1 is also expressed in high levels in healthy humans and correlates very well with CD45RO expression (*91*), therefore appearing to be important for memory differentiation. The link between PD-1 and disease progression arises from studies showing significant correlations between the levels of HIV RNA and CD4 count with PD-1 expression on activated T cells (*90, 92*). In conjunction, the expression of PD-1 has previously (*93, 94*) and in **paper 1** been defined as a good prognostic marker of absolute CD4 recovery pre-ART. In unpublished data from Okoye *et al* (*73*), blocking of PD-1 with antibodies induced proliferation of central memory CD4+ T cells and regeneration of CD4+ T cells. The blockage of PD-1 has also been shown to increase T cell migration (*95, 96*), which might be linked to specific expression of chemokine receptors like CCR5. This data implicates that PD-1 not only regulates T cell responses, but also might have a more profound role in the processes of T cell proliferation and migration that impedes normal homeostasis after HIV infection.

1.3.4.3 Immune aging (senescence)

Normal life progression is accompanied by a gradual aging of our immune system. This process is usually called immune senescence and is characterized by several features like shorter cell telomeres, increased CMV replication, low CD4/CD8 and naïve/memory ratio and many other variables (reviewed in (*83*)). Interestingly, many of these characteristics are also distinguishable in both chronic and treated HIV infection. Because of various events, potentially including increased CMV replication, T cells are driven to an end stage of their life cycle where the cells lose their ability to proliferate. Senescent T cells have previously been shown to express no CD28, but high levels of CD57 (*97*). Both untreated and treated individuals infected with HIV have a highly senescent T cell repertoire (CD28-CD57+), resembling much older healthy controls (*98*). Potentially, senescence of the T cell repertoire will generate problems for

individuals on long-term ART to efficiently respond to vaccine antigens. However, no direct correlation has been found between CD57+ or CD28- cells and CD4 recovery (**paper 1**). Thus, whether senescence is a cause or consequence of HIV disease progression remains obscure.

1.4 THE IMMUNE SYSTEM

1.4.1 Innate immunity

Innate immunity represents the first line of defense against pathogens and is usually known as the "non-specific" part of the immune system. This expression is related to the fact that no education of innate immunity is necessary before an encounter with a foreign antigen. Instead, within hours after infection, different parts of the innate immune system recognize pathogen associated molecular patterns on invading organisms. The pathogen associated molecular patterns are usually identified by pathogen recognition receptors, which are widely expressed on numerous different cells of innate immune system.

- **Granulocytes**
  Neutrophils, eosinophils and basophils all belong to the granulocyte group. These cells are also known as polymorphnuclear cells due to their specific lobed nuclei. The neutrophils are the most abundant white blood cells (leukocytes) in the body and constitute 50-60% of all leukocytes. Neutrophils are usually the cells that first recognize invading pathogens and launch a generic response to kill the pathogen. They recognize pathogens and kill them through release of cytotoxic substances and reactive oxygen species. Eosinophils are much less abundant than neutrophils, but also act through the release of reactive oxygen species to kill invading organisms. These cells are particularly important in the recognition of certain parasites (helminthes) and also contribute to allergic reactions. In conjunction, the basophils are also instrumental in the handling of parasite infections. Importantly, these cells are also major producers of histamine and therefore play a central role in numerous inflammatory reactions, like asthma and allergies.

- **Mast cells**
  These cells are usually present in the mucosa and connective tissues and are usually known for their importance in their recruitment of other immune cells and wound healing properties. The mast cells are major producers of histamine, which dilates the blood vessels and leads to recruitment of other immune cells.

- **Monocytes and macrophages**
  In the blood, there are specific phagocytic cells called monocytes. These are the largest leukocytes and constitute up to 10% of all white blood cells. Monocytes are only present in the blood and differentiate into dendritic cells (DCs) and macrophages when they enter peripheral sites of the body. Macrophages recognize pathogens through PRRs and are perhaps the most efficient phagocytes in the human body, where numerous invading pathogens are killed before the macrophages die themselves.

- **Natural killer (NK) cells**
  Natural killer (NK) cells are lymphocytes and kill tumor and pathogen-infected cells through a delicate balance between activation and inhibitory receptors on the surface of target cells. In general, however, NK cells recognize compromised host cells through the absence of MHC I molecules (also known as the "missing self"). Normal cells of the body have an intact repertoire of MHC I and are therefore not killed by NK cells. Recent evidence implicates that NK cells might have some kind of memory and are therefore usually regarded as something in between adaptive and innate immunity.

- **γδ T cells**
  In contrast to "normal" T cells expressing the αβ-T cell receptor (TCR), there is also an unconventional repertoire of T cells called γδ T cells, due to their expression of γδ-TCRs. These cells are also placed on the border between the adaptive and innate immune systems: Partly because they go through TCR rearrangement, but only recognize common patterns of pathogens through their γδ receptors. The role of γδ T cells is still widely debated, but recent research implicates that these cells might recognize non-peptides, including lipids of extracellular bacteria and viruses.

- **Complement system and antimicrobial peptides**
  Physical cells are not the only component of the innate immune system. The complement system consists of over 25 smaller proteins that are produced in the liver and then circulate in the blood as inactive precursor proteins. Due to specific reactions in the body, which usually involve inflammation, the complement precursors are cleaved into active proteins that through different cascades facilitate antibodies and phagocytic cells to clear infectious particles. Antimicrobial peptides are also smaller proteins that act at different sites of the body. These peptides can either create pores in the membrane or penetrate it to act within cells to kill for instance bacteria. However, in general the microbial peptides have numerous different roles in the immune response against pathogens, which essentially involves a large number of immunomodulatory functions (*99*).

1.4.2 Activation of adaptive immunity

Although innate immunity preferentially acts to dampen the initial establishment of pathogens, one of its most crucial roles curtails its ability to activate the adaptive immune system. The DCs are particularly important for this process and together with other cells (B cells, macrophages and epithelial cells of the thymus) are known as "professional" APCs. After an encounter with a foreign antigen, APCs migrate to nearby secondary lymphoid organs where they process the pathogen-derived proteins and degrade them into smaller peptides. This procedure is known as antigen processing and is essentially divided into two distinct pathways: one for the presentation of peptides on MHC I molecules and the other for MHC II peptide presentation.

MHC class I processing, also known as the endogenous pathway, is initiated by the degradation of proteins (antigens) into 8-11 mer peptides in the proteasome. The optimal peptides are then transported via ER to TAP, where they are loaded on to MHC I molecules, and finally transported to the surface where a CD8+ T cell binds through its TCR. Although APCs are crucial for the presentation of antigens to T cells, MHC I molecules are expressed on every nucleated cell in the body. This allows MHC I antigen processing to happen in virtually every cell, in order for the adaptive immunity to recognize intracellular pathogens. However, only memory CD8+ T cells are able to bind via the MHC class I complex on normal cells while the education (specificity) of CD8+ T cells only occurs after APC-mediated presentation. MHC class II, on the other hand, is only present on APCs, and peptide loading on these molecules usually takes place after extracellular pathogens have been endocytosed (the exogenous pathway). MHC class II molecules in their inactive form are situated in the ER, where a small protein (invariant chain; li) blocks other self peptides from binding to the MHC II binding site. The MHC II molecule then fuses with the endosome (containing the pathogen) and the invariant chain is cleaved into a smaller protein called CLIP. After that the MHC-DM molecule removes CLIP and replaces it with a peptide derived from the pathogen. The MHC class II complex is then transported to the cell surface where it binds to CD4+ T cell via its TCR. The MHC class II molecule has a more open structure compared to MHC class I, and therefore binds peptides of longer fragments (9-24 mers). In general, though, the optimal size of a MHC class II peptide is 15 amino acids. As both extracellular and intracellular pathogens can be recognized by CD4+ and CD8+ T cells, APCs can "cross-present" antigens by skipping specific steps in one of the pathways to enable peptide loading on MHC I or II molecules (*99*) (**Figure 4**).

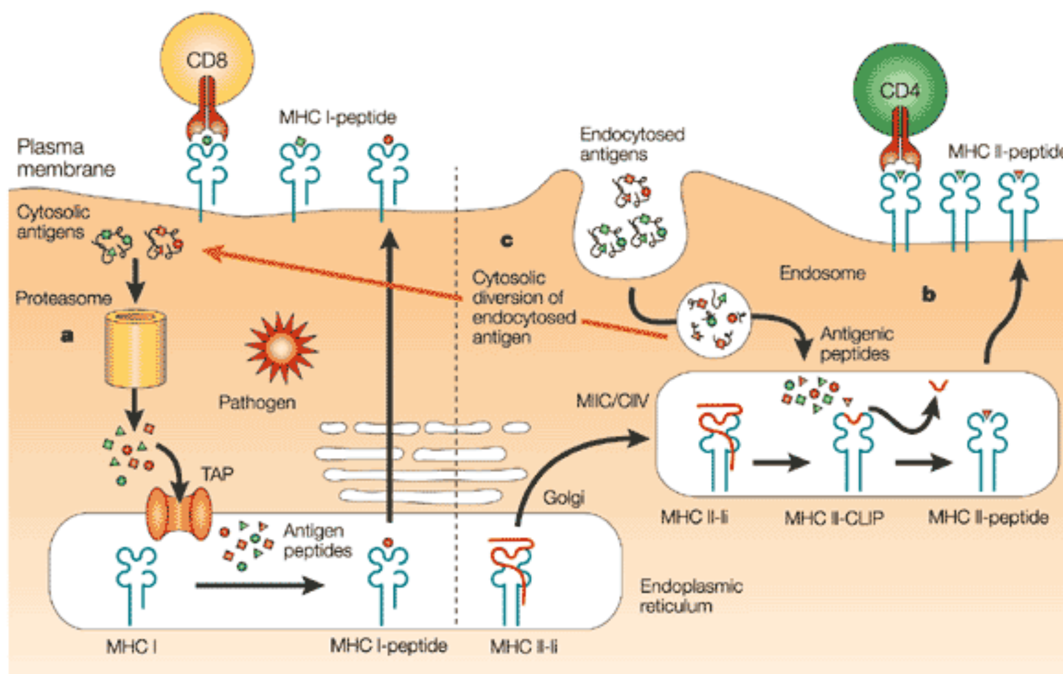


Figure 4. MHC class I and -II processing and presentation of antigens. Reprinted with permission from (*100*).

Once the peptides have been presented on MHC I and II molecules, naïve CD8+ and CD4+ T cells respectively bind through their TCRs to the MHC-binding complex. This

contributes to the education of the T cells and a polyclonal expansion of T cells that recognize a specific antigen. Although it might make sense that every individual recognizes the same antigens of specific pathogens, this is not the case. This stems from the vast polymorphism of the HLA alleles encoding the MHC I and II molecules. HLA-A, -B, and -C encode the MHC I molecules, while HLA-DR, -DQ and -DP code for the MHC II molecules. At present, thousands of different HLA alleles have been identified in the HLA database (http://www.hiv.lanl.gov/content/index) and contribute to the broad specificity humans possess to combat different pathogens. Humans encode between 3-6 HLA alleles depending on whether the individual is homozygote or heterozygote for a specific HLA allele. All HLA alleles code for specific MHC molecules with different binding motifs for their antigens, therefore contributing to diverse T cell specificity existing between different humans. However, while some of the HLA alleles might be associated with protection from disease after encounters with different infectious agents, others might increase the chance to develop autoimmune disorders due to presentation of specific self peptides to T cells (*99*).

1.4.3 CD4+ T cells

The central role of CD4+ T cells in human immunity has become particularly apparent after the HIV pandemic. CD4+ T cells orchestrate different arms of the immune system by essentially providing "helper" mechanisms to maintain normal immune-homeostasis. CD4+ T cells have therefore also been known as T helper (Th) cells, and possess an extreme plasticity making them prone to differentiating into diverse lineages of Th cells[#]. Initially, it was essentially accepted that CD4+ T cells were either Th1 or Th2 cells, which promoted CD8+ T cell or B cell responses respectively. However, with the introduction of new techniques dissecting the transcriptional regulation of specific cell types, numerous other Th lineages have emerged like Th9, Th17, Th22, T follicular helper (Tfh) and T regulatory (Tregs) cells (*101*).

Following the presentation of a foreign antigen from APCs, CD4+ T cells differentiate into diverse lineages of Th cells depending on several characteristics of the invading pathogen. Depending on the strength (avidity) of the TCR-peptide-MHC class II complex, local inflammatory milieu and expression of co-receptors, the CD4+ T cell will develop into a specific Th lineage. However, the specific Th profile is not locked and due to their plasticity, Th cells might turn into one another depending on the inflammatory milieu. The specific lineages of Th cells take their names fundamentally from their secretion of specific cytokines, which is due to expression of specific transcription factors. Th1 cells express the T cell specific T-box transcription factor T-bet, which promotes particularly IFNγ, but also TNF and IL-2 production. The secretion of these cytokines provides helper mechanisms for mobilization and migration of particularly CD8+ T cells, but also macrophages and other parts of the immune system. Th2 represent the other early-described Th cell lineage and express the transcription factor GATA3. This leads to the secretion of IL-4, IL-5 and IL-10, which are essential for class-switching of antibodies in order to generate proper humoral immunity. However, in recent years another Th cell type (Tfh cells) residing within the germinal center of lymph nodes has emerged as the central cell type to induce B cell maturation and somatic hypermutations, which is fundamental for an effective B cell response. Tfh have been defined based on the expression of the transcription factor B

cell lymphoma 6 (Bcl-6), which is of importance for the cells to primarily produce IL-21. This cytokine has an important role in both the activation and memory formation of B cells and CD8+ T cells, making the Tfh cells of great interest for future vaccine research in the field of infectious diseases. Th17 is another fairly newly discovered Th cell type, which primarily produces IL-17, but also IL-6, TGF-β and IL-1β. These cells have been described as expressing the RAR-related orphan receptor γt (ROR-γt) and recruit neutrophils and other cell types to the sites of infections, like mucosa, to protect the host from invading organisms. In contrast, Tregs have the opposite role in comparison with the other cell types, actually suppressing immune function. These cells express the transcription factor forkhead box P3 (FoxP3), enabling the cells to produce IL-10 and TGF-β (reviewed in (*101-103*)).

[#] In this thesis, “Th cells” will only be used when discussing specific lineages of Th cells. Otherwise, “CD4+ T cells” will be used as the general nomenclature for all lineages of Th cells.

1.4.4 CD8+ T cells

The other arm of T cell immunity consists of CD8+ T cells. These cells are more generally known as cytotoxic T cells, but CD8+ T cells have different roles depending on their differentiation status.

Following antigen presentation, CD8+ T cells undergo polyclonal expansion and differentiation in order to limit pathogen replication through direct killing of the infected cell. As previously described, CD8+ T cells recognize primarily intracellular pathogens (like viruses) through the presentation of pathogen-derived peptides on MHC class I molecules on target cells. The binding of CD8+ T cells to the MHC-peptide synapse leads to the release of cytotoxins, which are present within the granules of CD8+ T cells. Different cytotoxins have been shown to induce cell death, including perforin, granzyme A, B, K and granulysin. However, only perforin and granzyme B are seen to be associated with target cell lysis in cell cultures of virus-specific CD8+ T cells and target cells (*104-106*). Perforin is a cytoloytic protein, which forms pores in the membrane of the target cell, essentially leading to direct lysis of cells and/or the passage of Granzyme B into the cell. Granzyme B is a serine enzyme, which catalyzes the cleavage of specific substrates (particularly caspases) in the process of programmed cell-death (apoptosis). Together, these actions leads to cell death and the inability of intracellular pathogens to replicate and therefore survive (*99*).

In order for the cells to differentiate into specific CD8+ T cells with cytotoxic or more regulatory functions, two transcription factors are of central importance: the T-box transcription factors T-bet and Eomesodermin (Eomes). In the acute phase of an infection, T-bet and Eomes cooperate in order to induce a massive expansion of effector cells to clear the infection (*107-109*). If the infection is eradicated, T-bet expression usually declines while Eomes is gradually up-regulated as memory CD8+ T cell homeostasis is maintained to launch an effective secondary response in case of re-infection (*110-113*). Several studies have thus suggested that the expression ratio between T-bet and Eomes determines the terminal differentiation or long-term survival of CD8+ T cells (*114*). Within memory T cells, T-bet promotes primarily the

expression of proteins linked to cytotoxicity, like perforin and granzyme B expression, but also other functions including IFNγ and TNF secretion. Therefore, T-bet promotes the generation of CD8+ T cell differentiation, and these cells are usually called effector and effector memory cells depending on their expression of certain phenotype markers. These cells tend to reside in the periphery, due to expression of CCR5, and have less CD27 and lymph node homing markers (*115*). Eomes, on the other hand, enables cells to compete for the less differentiated memory cell niche (*110*). Down-regulation of Eomes leads to defects in long-term survival and secondary expansion, which are all attributes of central memory cells. These cells usually express the lymph node homing markers (CCR7 and CD62L) and CD27, making them prone to circulate between blood and lymph nodes and produce IL-2. The long-term persistence of these cells is probably due to the expression of the IL-7 receptor (CD127), which confers survival signals to these cells (**Figure 5**). Overall, however, T-bet and Eomes cooperate to contain chronic infections, and without either subset an imbalance of CD8+ T cell differentiation might occur, leading to the inability to clear pathogenic infections (*116*) (**paper 4**).

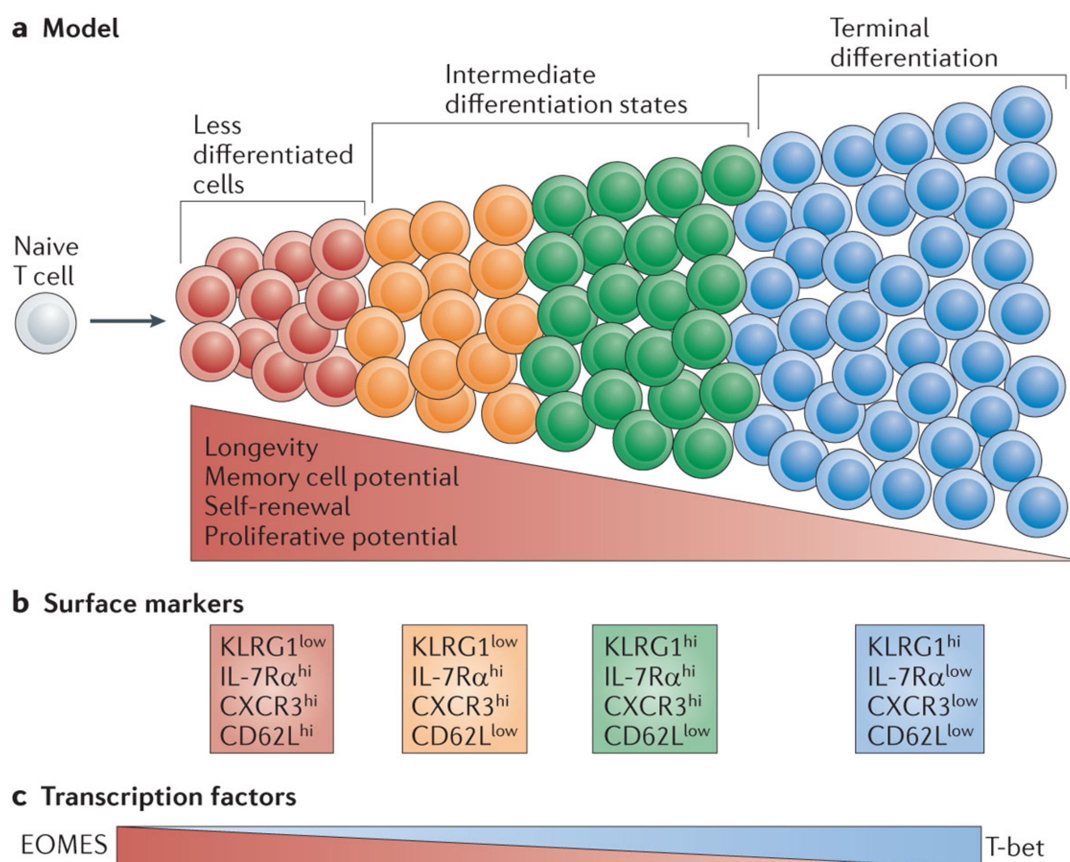


Figure 5. Impact of T-bet and Eomes on CD8+ T cell differentiation. Reprinted with permission from (*114*).

1.4.5 B cells

Antibodies are instrumental for almost every vaccine we know of today. The production of antibodies is mediated by B cells, that are created in the bone marrow but migrate to the secondary lymphoid tissues where activated. All B cells are unique due to their expression of B cell receptors on their surfaces. The B cell receptor is a membrane-bound immunoglobulin (Ig) molecule that binds to specific antigens. After

cognate antigen binding and signaling from Tfh and Th2 cells, the B cells generally differentiate into plasma or memory B cells. However, several intermediate B cell differentiation steps can also take place, in which cells undergo somatic hypermutations in the germinal center and possibly also class-switching into specific isotypes like IgD, IgG, IgM, IgE and IgA.

The plasma cells are the major producers of antibodies. Plasma cells correspond to effector T cells in different ways: They both execute their effector functions and are short-lived. The produced antibodies either bind to a specific antigen and thereby neutralize the pathogen directly, or otherwise bind to the antigen and through its Fc receptor bind to other cell types to mediate engulfment, through a process called antibody-dependent cell-mediated cytotoxicity (ADCC). The memory B cells, on the other hand, are smaller cells, and possess antigen specificity against the pathogens encountered during the primary phase of infection. Most commercial vaccines today are based on eliciting a good memory B cell response, as these cells are long-lived and quickly respond after a secondary challenge with the specific antigen (*99*).

1.5 HIV-specific immunity

1.5.1 Correlates of disease progression

The rate of HIV disease progression is determined by numerous factors involving both viral and host interactions. Correlates of HIV disease progression have specifically been studied in a group of subjects that do not progress to AIDS despite lack of ART. These individuals are usually called long-term non-progressors (LTNPs) and constitute a small proportion of individuals (5-15%) with low to moderate levels of HIV RNA, which remain immunologically stable for numerous years after infection. Some of these individuals also possess undetectable viremia for a longer consecutive period of time without any ART. These subjects are usually called elite controllers and comprise less than 1% of all HIV infected subjects (*117*).

Undetectable viral load was first demonstrated in the mid-1990s when it became possible to measure the HIV RNA levels in plasma. Initial studies showed that isolates of HIV lacking Nef generated replication-defective viral particles, and therefore implied that attenuated viral particles were the cause of undetectable viremia in individuals off ART (*118*). However, most of these studies were conducted on small sample sizes, with poor controls and assessment of viral sequences instead of replication-competent viruses in individuals with low, but not undetectable viremia (*117*). Later, when the high sensitive co-culture assay was developed, it became apparent that elite controllers had replication-competent viruses without any insert deletions (*119*). A later study demonstrated that an individual that developed full blown AIDS transmitted HIV to another person that became an elite controller (*120*), which further indicate that these individuals are infected with highly pathogenic strains of HIV.

Most recent studies have suggested that the rate of disease progression and viral control is rather influenced by the host immune system. After discovering CCR5 as the co-

receptor of HIV, individuals with a 32 base pair deletion within the CCR5 gene (*CCR5Δ32*) were shown to be protected against acquisition of HIV (*CCR5Δ32* homozygosity) or development of AIDS (*CCR5Δ32* heterozygosity) (*121-126*). Likewise, individuals with a higher copy number of *CCL3L1* (the gene encoding the CCR5 ligand MIP-Iα) generally have lower HIV viremia (*127*), although a complex interplay between variants of *CCL3L1* and *CCR5* works to hamper HIV replication (*128*). Although genes for HIV entry might be of importance for disease progression, most of the close genetic correlates of HIV control involve the expression of specific HLA alleles. In a recent report, specific HLA-DRB1 alleles were associated with low viremia (*HLA-DRB1*1502*) and high viremia (*HLA-DRB1*0301*), suggesting that HIV-specific CD4+ T cells might play an active role in the containment of viral replication (*129*). In addition, specific gene subsets involved in HIV outcomes have been linked to NK cell engagement, where subjects expressing the active KIRDS1 allele together with Bw4-80l (MHC class I ligand) will in particular progress slowly to AIDS (*130*). However, though the entire immune system plays some role in hampering HIV replication and delay disease progression, HLA I alleles are most noticeably correlated with the outcome of HIV disease (reviewed in (*131*)). In the recent HIV controllers study (*132*), more than 1 million single-nucleotide polymorphisms were obtained from 974 controllers and 2648 progressors. Over 300 single-nucleotide polymorphisms were identified as significantly different between the groups, where all were located within the MHC region. Using regression models, several alleles were associated with protection (*HLA-B*5701, B*2701, B*14/Cw*0802, B*52 and A*25*) or risk (*HLA-B*35 and Cw*07*) of disease progression. More recently, the expression levels of HLA-C on the surface cells have also been correlated with delayed progression to AIDS (*133*), implicating that it is not only specific HLA alleles which confer protection, but also the quantity of these alleles that are expressed on target surfaces.

Consistent to most other reports, HLA-B57 came out at the top in the HIV controller study of alleles linked to host protection against HIV disease. How HLA-B57 enhances control of HIV is not entirely known, although the recognition of a broad number of peptides from conserved regions (*134, 135*), which generate high magnitudes of HIV-specific T cells (*135, 136*), might be a rational explanation. Another open question is whether or not there is a crosstalk with the innate immune system as HLA-B57 directly binds to certain killer-immunoglobulin receptors. However, it seems likely that HLA-B57 subjects develop into LTNPs or elite controllers due to peptide-binding flexibility during the thymic development (*137*). It has been suggested that HLA-B57-restricted T cells are cross-reactive against mutants due to the recognition of fewer self-peptides during the negative selection process in the thymus. Similar mechanisms have been implicated for the protective effects of HLA-B27. The model linking less stringent negative selection and cross-reactivity of peptides is also an attractive one in autoimmunity, as both HLA-B57 and –B27 confers increased risk to develop these disorders (*138, 139*). However, despite most studies having linked host genetic factors with HIV disease progression, only a small fraction of *e.g.* all HLA-B57+ subjects develop into LTNPs or elite controllers. Whether this is due to the clonal composition of the T cell repertoire remains contested (*140, 141*). Therefore, the process of disease progression is most probably influenced by both viral and host factors after HIV transmission (**Paper 3**).

1.5.2 HIV-specific CD4+ T cells

Despite being the central target for HIV virions, the role of CD4+ T cells recognizing HIV-derived peptides (HIV-specific CD4+ T cells) still proves elusive for the field of HIV-specific immunity. Primarily, this is because of the inability to ascertain whether the presence of HIV-specific CD4+ T cells is a cause or consequence of HIV disease progression itself. Nevertheless, even though HIV-specific CD4+ T cells are primarily infected with the virus (*142*), early preservation of these cells has been associated with viral control (*143*). HIV-specific CD4+ T cells are generally induced during early infection, and the magnitude of the responses peak at 1 month post infection (*144*). However, these responses still tend to be quite low, and studies have shown that HIV-specific CD4+ T cells become impaired and lose proliferative abilities early post infection (*145*), potentially due to loss of IL-2 production and not by physical depletion by HIV itself (*146*). Individuals who experience the highest or most vigorous HIV-specific CD4+ T cell responses tend to be those with poor treatment adherence/low-level ART resistant viremia (*147*) or elite controllers (*148*). The latter group also experiences increased functional avidity (*149*), proliferation (*148, 150*), and increased polyfunctional characteristics (measured by the fraction of cells producing IFNγ, TNF, IL-2 and MIP-Iβ simultaneously) (*151, 152*). However, increased IL-2 production, proliferation and diverse phenotypic markers have also been attributed to ART-treated subjects, indicating that these characteristics are a consequence, rather than a cause, of lower HIV viremia (*153, 154*).

Gag- and Nef-specific cells dominate the total pool of HIV-specific CD4+ T cells (*155*). In particular the breadth of Gag-specific CD4+ T cells has been associated with lower viral load in both adults (*156*) and children (*157, 158*). In one of these studies, Ranasinghe *et al* distinguished that Env-specific responses were highly present in chronic progressors, while elite controllers experienced an inverted Env-/Gag-specific CD4+ T cell ratio. This data would suggest that targeting of conserved proteins by CD4+ T cells might establish pressure on the founder virus. One of the questions that remain, then, is how broad Gag-specific CD4+ T cell responses might be established. Streeck and colleagues recently demonstrated that specific HLA-DRB1 molecules are independently associated with lower or higher viral load (*129*). Specifically, the HLA-DRB1 alleles associated with lower viremia were able to present a broad number of Gag and Nef peptides, indicating that the breadth of MHC class-II restriction might confer protective effects against HIV disease progression. Interestingly, in a recent report where a replicating CMV-vector containing SIV genes was used, extremely broad CD8+ T cell responses were detected. Two-thirds of the responses were shown to be MHC class II-restricted, which implicates that effective responses hampering pathogenic lentiviruses can be achieved through the broad capacity of MHC class II to present peptides (*159*).

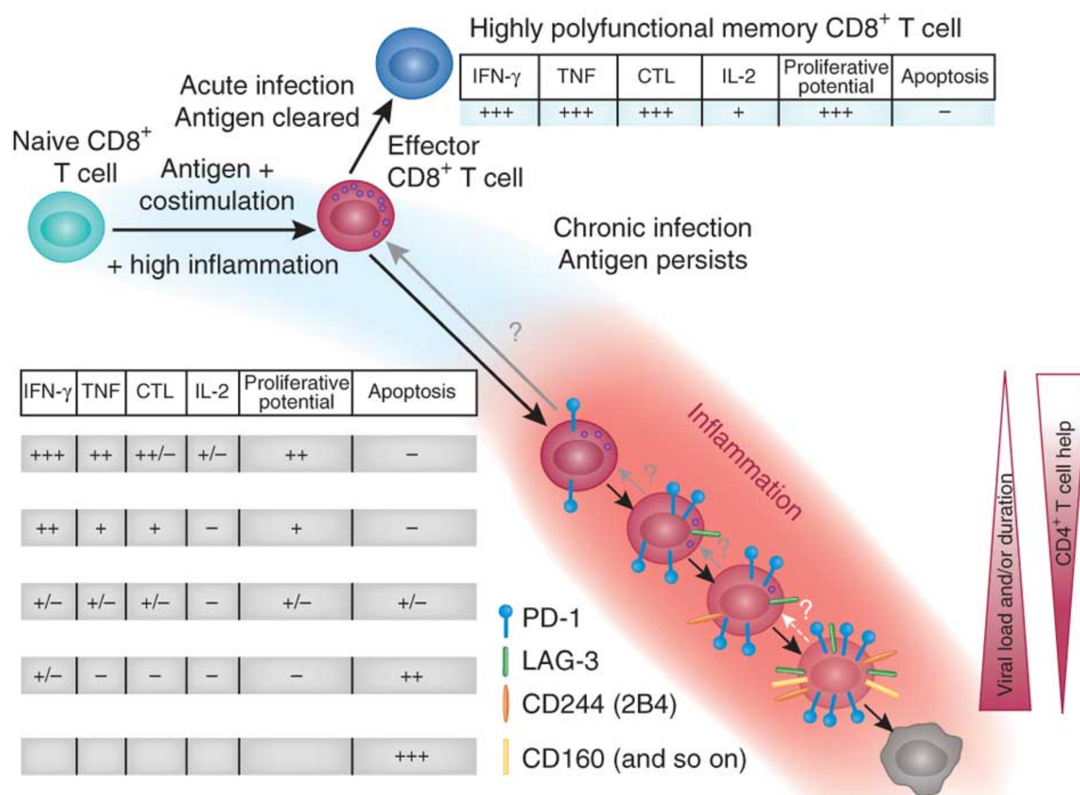
1.5.3 HIV-specific CD8+ T cells

HIV-specific CD8+ T cell responses represent a major factor in predicting the outcome of HIV disease progression. The pivotal role of CD8+ T cells was first demonstrated in 1994, when two seminal studies showed a close association in early infection between the increase of HIV-specific CD8+ T cells and the dramatic decrease of viral load (*160,*

161). It was later confirmed that depletion of CD8+ T cells, through supplementation of anti-CD8 antibodies, in SIV-infected macaques resulted in a drastic increase of viremia and death of the macaques (*162-164*). The development of viral mutations has primarily been detected within MHC class I-restricted epitopes (*52, 56, 165-167*), and coincides with the reduction of viremia (*168*), which further suggests the important role of HIV-specific CD8+ T cells to elicit pressure on transmitted founder viruses.

CD8+ T cells are directed against HIV early after infection, peak around 300 days post infection and then stay elevated throughout the course of disease (*144*). Importantly, though, the magnitude of the CD8+ T cell response is not the sole determinant of HIV disease protection, and actually positively correlates with HIV viremia (*169*). Therefore, specific functional characteristics (the quality) of CD8+ T cell responses have been investigated thoroughly instead. In former studies, primarily MIP-1β and IL-2 production have been shown to exert non-cytolytic antiviral effects to potentially hamper viral replication (*170, 171*). From a cytotoxic perspective, increased perforin (*172, 173*) and Granzyme B (*105*) production has been demonstrated in LTNPs, which might be a consequence of increased T-bet expression (*173*). Although specific functional characteristics might posses an important role, numerous studies have shown associations between polyfunctionality and viral control (*174, 175*). Qualitative features surely represent an important part of an effective immune response, but most of these studies have been conducted in cross-sectional settings. Thus, it remains uncertain which are the causes and consequences of CD8+ T cell responses putting selective pressure on autologous viruses.

Because of evasion from the immune system, HIV turns into a chronic disease that is thought to result in an exhausted pool of CD8+ T cells (reviewed in (*176*)). Exhaustion of CD8+ T cells is characterized by a gradual loss of different functions that initially includes a lack of possibility to proliferate (produce IL-2) and induce killing (up-regulation of cytotoxins). At a later phase, TNF is typically diminished and finally the ability to produce IFNγ or degranulate is generally lost (*177, 178*). Murine studies initially revealed that chronic lymphocytic choriomeningitis virus clone 13 (LCMV-13) infection causes an up-regulation of PD-1 (*179*) and other inhibitory receptors, including CD160, 2B4 and Lag-3 acting cooperatively to induce CD8+ T cell dysfunction (*180*). These findings were largely corroborated in later studies of human chronic infections including HIV (*86-88, 181*). Concurrently, HIV-specific CD8+ T cells have been demonstrated to have an intermediate (*182*) and skewed (*183*) maturation phenotype, which is not correlated with markers of T cell senescence (*184*). Thus, together these studies suggest that exhaustion and poor functional characteristics of HIV-specific CD8+ T cells might not be due to extensive cell-cycle progression, but rather an intermediate differentiation phenotype due to specific transcriptional regulation (**Figure 6**) (**paper 4**).



IFN-γ	TNF	CTL	IL-2	Proliferative potential	Apoptosis
+++	++	++/–	+/–	++	–
++	+	+	–	+	–
+/–	+/–	+/–	–	+/–	+/–
+/–	–	–	–	–	++
					+++

Figure 6. Attributes of CD8+ T cell exhaustion. Reprinted with permission from (*176*).

1.5.4 The design of vaccine antigens

Although ART and other preventive actions have had tremendous impact on transmission and morbidity, an effective vaccine will probably be the final action to durably control and end the HIV pandemic. However, the field of HIV vaccinology has been rife with disappointments for a very long time. The first larger vaccine trial took place already in 1986, where an *env* gp160 subunit vaccine was developed by MicroGeneSys to induce neutralizing antibodies. This simple vaccine was designed after the assumption that only the envelope would be needed to generate protective responses, as in the Hepatitis B vaccine that had recently been developed with great success. However, the *env* gp160 subunit vaccine trial failed and showed that normal subunit vaccines do not generate broadly neutralizing antibodies (bNAbs) against primary isolates of HIV (*185*). Later, in 2003, VaxGen developed a recombinant gp120 vaccine thought to induce broader antibody responses against HIV. However, like the previous antibody-mediated vaccine, this trial also failed to induce any response (*186*). Subsequently, the field started to show a greater interest towards generating T cell based vaccines using mainly recombinant viral vectors, DNA vaccines and combinations of heterologous vaccines through prime/boost regimens (*185*). However, in 2007 (STEP trial) (*187*) and 2013 (HVTN 505 trial) (*188*), two recombinant Adenovirus 5 vector vaccines did not demonstrate any correlation of protection and rather generated increased risks of HIV transmission. After years of disappointment, some hope was restored when the RV144 trial proved to reduce the risk of HIV transmission with 31% (*189*). Despite most of the correlates later having been linked to the development of antibodies against certain regions of the HIV envelope, this vaccine

was based on the combination of generating B and T cell responses, clearly demonstrating that a combination of both arms of adaptive immunity most probably will be needed for future candidates to be successful.

The modest efficacy of the RV144 trial has surprisingly been correlated with the development of non-neutralizing antibodies targeting the V1/V2 region of gp120 (*190, 191*). As these antibodies have not been demonstrated to neutralize HIV by nature, this data implicates that antibodies mediated against this region potentially lead to ADCC. In contrast to non-neutralizing antibodies, the field of antibody research has mostly been interested in studying bNAbs. During recent years numerous bNAbs against HIV have been described (reviewed in (*192*)), mostly targeting conserved regions of the envelope. In two recent studies, passive transfer of numerous bNAbs was shown to protect against challenges with specific SHIV strains (a chimeric SIV/HIV virus). Although the generation of bNAbs might be a golden example of a future HIV vaccine candidate, numerous obstacles still exist. For instance, the conserved parts of the envelope are highly glycosylated, making them poorly immunogenic. In addition, the elicitation of bNAbs is only seen in a small number of patients and usually develops numerous years (>2 years) post-infection. Most of the bNAbs demonstrate extensive somatic hypermutations and could possess autoreactive traits as well (*193, 194*). A recent study conducted longitudinally from a HIV infected individual suggested that high degrees of somatic mutations and bNAb development were associated with extensive evolution of the transmitted founder virus (*195*). All these features together suggest that elicitation of bNAbs with one specific antigen will probably not induce bNAbs under a long period of time and therefore new strategies might be needed. Potentially, this will include an iterative design of vaccine antigens that mimic the evolution of the HIV envelope in infected subjects (*192*).

In order to induce long-lasting immunity (including bNAbs), HIV-specific CD4+ T cells might hopefully play an important role. The induction of these cells has been avoided due to their preferential infection with HIV (*142*), but new studies have suggested that these cells might be of high relevance in future vaccine regimens. The RV144 trial identified specific MHC class-II restricted responses and high Env-specific CD4+ T cell responses in those subjects with decreased risk (probability) of infection (*190*). Recently, Tfh helper cells, which reside in the secondary lymphoid organs and induce B cell maturation, have received increased interest. Tfh cells are known to enhance the potency of NAbs by increasing B cell somatic mutations and in a recent report, the frequency of Tfh cells was higher in a large number of subjects that developed bNAbs (*196*). However, recent studies have also shown an increased frequency of Tfh in HIV and SIV infection that was associated with the extreme hypergammaglobulinemia seen after infection (*197-199*). Thus, although the Tfh cells might play a crucial role for the development of bNAbs, further studies need to delineate how to induce a balanced Tfh response in order to avoid antibody responses of poor specificity against HIV.

As demonstrated both in the STEP and HVTN 505 trial, CD8+ T cell responses elicited by Adenovirus 5 vectors have not been sufficient to induce protective effects against either HIV transmission or disease progression. However, the responses mediated by the Adenovirus 5 vectors were quite narrow, targeted primarily variable regions like

Env (*200*) and only individuals with protective HLA alleles seemed to induce cytolytic CD8+ T cell responses facilitating protection against disease progression (*201*). The limited breadth and vaccine efficacy elicited by the Adenovirus 5 vectors have recently been demonstrated by Louis Picker's group, which compared numerous vaccine candidates versus their own replication competent CMV-vectors in rhesus macaques (*202*). The SIV-expressing CMV-vectors induce persistent effector memory CD4+ and CD8+ T cell responses that elicit effector functions at peripheral sites of the body immediately after antigen encounter (*203*). Importantly, the CMV-vectors induce extremely broad cellular responses that violate all paradigms of antigen recognition known about so far (*159*). The CD8+ T cell responses are both MHC class I and II-restricted, which potentially mediates the clearance of pathogenic SIV that has been distinguished in monkeys several years after viral challenge. These different studies suggest that CD8+ T cell responses could potentially protect and even clear HIV, but there are still several obstacles in the way of their employment in vaccine regimens. First, high quantities/frequencies of HIV-specific CD8+ T cells, potentially targeting multiple epitopes, need to be elicited for a long period of time. Antigen-specific cellular responses tend to decline over time if the antigen is cleared and therefore heterologous prime-boost vaccination strategies need to elicit enough quantities over a persistent-period of time. CMV-vectors are replicating vectors, and the long-term effects of these vectors on the immune system are unknown; particularly as CMV has been thought to drive immunosenescence. A broad response targeting multiple epitopes might be another rationale for future cellular vaccines, potentially through mosaic antigens (*204*) to impede immune escape from MHC class I and II-restricted responses. Whether an effector memory CD8+ T cell response is needed is another highlight of current discussions. The central memory CD8+ T cell response, usually comprising a large proportion of existing memory cells, resides in the lymph nodes and the activation of these cells happens when HIV has breached the systemic walls. Thus, effector memory responses probably need to be primed for a direct anti-HIV response. Finally, the peripheral localization of the response also remains of importance as HIV most commonly is transmitted though sexual routes. However, if it becomes possible to induce persistent effector memory T cells, the underlying transcriptional regulation will also facilitate peripheral migration of these cells through chemokine receptor expression. For instance, T-bet has been shown to facilitate both effector memory responses and migration to periphery sites of the body through chemokine receptor regulation (*115*). Therefore, integrated studies of basic and translational science will hopefully bring us closer to induce HIV-specific CD8+ T cell responses of high quantity, quality and localization that potentially will impede viral replication before systemic contamination of the virus occurs (*205*).

2. AIM

The aim of my thesis was to combine immunological methodology with bioinformatics to characterize T cell pathogenesis and function in HIV infected individuals.

Specific aims:

Paper I: To determine which routine laboratory parameter is most strongly associated with combined T cell pathology, using multi-parametric flow cytometry and advanced bioinformatics.

Paper II: To develop and evaluate a novel epitope selection algorithm that identifies optimal MHC class II-restricted HIV epitopes with broad viral and host coverage.

Paper III: To investigate whether the combined pattern of HIV evolution and CD8+ T cell functionality could explain the risk of HIV disease progression in HLA-B*5701+ patients.

Paper IV: To clarify the role of T-bet and Eomes in CD8+ T cell dysfunction after HIV infection using polychromatic flow cytometry and bioinformatics.

3. MATERIAL AND METHODS

3.1 CLINICAL MATERIAL

The patient material in **paper I** consisted only of peripheral blood mononuclear cells (PBMCs). These samples were collected from 47 HIV-infected individuals from the Outpatient HIV Clinic at Karolinska University Hospital Huddinge, Stockholm, Sweden. The PBMCs were isolated from whole blood by Hypaque-Ficoll density gradient centrifugation and cryopreserved before staining and flow cytometry analysis. HIV-infected subjects in Sweden are a quite heterogeneous group (*206*), as is illustrated in this study, where a fairly high percentage of the subjects were of African or Asian origin (38%). An age- (median: 39 years) and sex- (62% male) matched healthy control group of 21 individuals was also recruited from Karolinska Institutet to compare all of the immunological markers (*207*).

The patient material in **paper II** was also acquired in Sweden, where both fresh PBMCs (for enzyme-linked immunospot, flow cytometry analysis and HLA typing) and plasma (for HIV sequencing) samples were collected for immediate immunological analysis. This study cohort consisted of 38 HIV-infected individuals with ten different HIV-1 subtypes. The study participants were recruited from the Outpatient HIV Clinic at Karolinska University Hospital and Stockholm South General Hospital, Stockholm, Sweden. Patient inclusion was based on the single criteria of a CD4+ T cell count >300 cells/ mm^3 to ensure detection of HIV-specific CD4+ T cell responses. Of the included patients, 25 were treated (viral load <50 copies/ mL, except for one individual with poor treatment adherence) and 13 untreated (viral load >50 copies/mL) (*208*).

The patient material in **paper III** was collected from the OPTIONS cohort at University of California San Francisco, USA. The OPTIONS project is a multi-study research program where individuals are followed longitudinally from early HIV infection (within 6 months of HIV seroconversion). Both plasma (single-genome-sequencing) and PBMCs (flow cytometry) were acquired from six HLA-B*5701+ HIV subtype B-positive men. All subjects were followed from early infection up to seven years (for a similar number of weeks) while they remained untreated (*209*).

The patient material in **paper IV** was gathered at the Outpatient HIV Clinic at Karolinska University Hospital Huddinge and Venhälsan at Stockholm South General Hospital (Stockholm, Sweden). In total, 52 individuals with chronic untreated HIV infections and 12 HIV-infected individuals on ART for more than 10 years (fully suppressed viral load for >8 years) were enrolled in this study. Cell samples from 20 healthy controls were also collected from the Karolinska Institutet and Karolinska University Hospital Huddinge. Out of the 52 individuals with chronic untreated HIV infection, 24 individuals were followed longitudinally from baseline (median = 0 days before ART initiation) and at 2 weeks, 4 weeks, 8 weeks, 12-16 weeks and 5-7 months post-ART. All individuals followed longitudinally initiated ART in the chronic phase of HIV infection and were treated successfully with no detectable HIV viremia after 5-

7 months on ART.

3.2 ETHICAL CONSIDERATIONS

All study participants were provided with written and oral information about the studies. Written and oral informed consent was documented from all study subjects in accordance with the Declaration of Helsinki. The Regional Ethical Council in Stockholm, Sweden approved all studies. For **paper III**, The University of California, San Francisco (UCSF) Committee on Human Research also approved the study.

3.3 LABORATORY METHODS

3.3.1 Flow cytometry

In order to characterize the traits of T cell pathology and functionality, all studies involved multi-parametric flow cytometry analysis. Flow cytometry, or fluorescent activating cell sorting (FACS), is a method that is based on the ability of cells to react differently to light. Thousand of cells are lined (fixated) per second in a stream and pass through a laser beam one at a time. This generates information of specific cell characteristics on a single cell level, which essentially is the great advantage with FACS. Thus, instead of receiving information from a bulk cell population, FACS allows detailed information of a vast number of cells. The generation of high dimensional data on single cell level is primarily due to the usage of fluorescence-conjugated monoclonal antibodies, which allows for detection of different markers simultaneously on specific cell populations. During the past few years numerous new fluorochromes have been developed, including the bright Brilliant Violet colors (*210*), that enable increased detection of specific cell populations, which were impossible to delineate before (**Figure 6**).

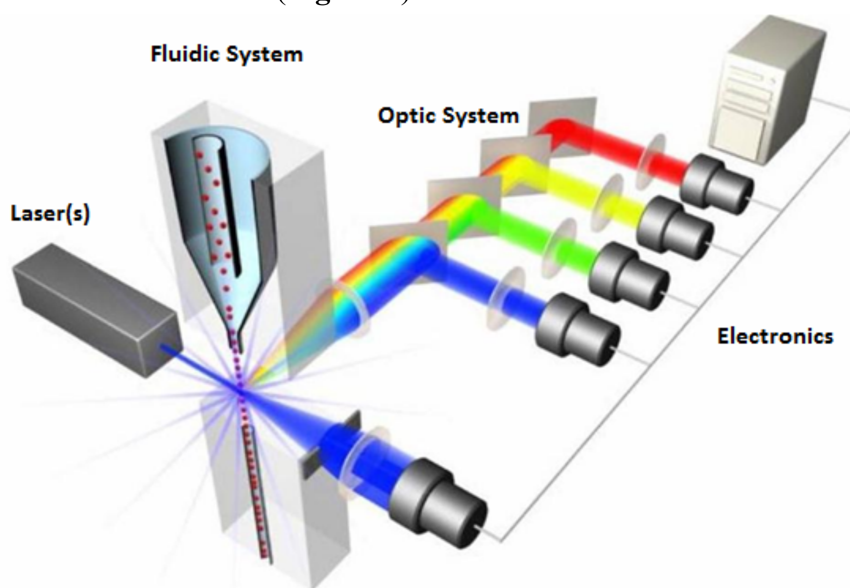


Figure 6. Schematic picture of basic components in a flow cytometer.

3.3.1.1 Peptide stimulations and intracellular stainings

For most of the studies, **papers II-IV**, intracellular stainings with fluorescent-conjugated monoclonal antibodies were used to detect cytokine, chemokine, and cytotoxin expression after peptide stimulations, and/or other intracellular proteins (including transcription factors). Staining protocols for detection of intra-cellular or intra-nuclear proteins differs in respect to fixatives and permeabilizing agents.

Peptide stimulations are generally performed to detect any antigen-specific T cell response in the cell culture systems. The peptide stimulations in **paper II** took place on fresh cells, as peptide immunogenicity with this material had previously been demonstrated to increase the detection sensitivity of HIV-specific CD4+ T cell responses. The detection sensitivity of HIV-specific CD8+ T cell responses are almost equal using fresh or cryopreserved PBMCs (*211*). Therefore, for **papers III-IV** peptide stimulations of CD8+ T cells were assessed using cryopreserved PBMCs, which, were thawed and rested overnight before usage (*211*). In order to detect antigen-specific production of cytokines in cells, peptides are added to the cell cultures. This involves the administration of agents (including Brefeldin A) that inhibit transportation from ER to the Golgi apparatus by blocking the formation of transport vesicles. Brefeldin A thereby disenables cytokines to be secreted from the cells and they instead accumulate inside the cytosole. The peptide stimulations range from 6-10 hours **(papers III-IV)** for the detection of HIV-specific CD8+ T cell responses to 12 hours (**paper II)** for the detection of HIV-specific CD4+ T cell responses. Differences in time stem from the diverse time-period for antigen processing and presentation to take place for MHC class-I and -II restricted responses, respectively.

In **paper I**, extracellular surface staining was used, which generally only entails a thawing step of cryopreserved cells before incubation with fluorescent-conjugated monoclonal antibodies directed against proteins on the cell surfaces. However, to detect intracellular proteins, a second step is necessary after the extracellular incubation with antibodies. In **papers II-III,** normal fixatives and permeabilization agents were used to enable antibodies to enter the cytosole. This allows for the detection of cytokines and other proteins present in the cytoplasma. In order to detect the expression of transcription factors, however, other fixatives and permeabilizing agents are necessary to enable monoclonal antibodies to enter the nucleus (**paper IV**). Previous studies have found that it is possible to use normal fixatives and permeabilization agents to detect transcription factors, but we have distinguished improved signals by the usage of transcription factor fixatives. Importantly, these fixatives do not change other cell characteristics, including cell surface markers, and it is still easy to detect cytokines in the cytosole.

In **paper IV**, we also wanted to determine whether the overlapping peptide stimulations generated consistent results with unstimulated/resting cells and, therefore, HIV- and CMV-specific CD8+ T cells were subsequently identified with MHC class-I tetramer stainings. MHC class I tetramers are bound to a specific peptide and mimics antigen-presentation, which allows for direct assessment of protein expression in/on

cells that have not been stimulated with antigens. To make a matching experiment, we screened for HIV-infected donors, identified in a previous study (*206*), that were HLA-A*0201+ and had CD8+ T cell clones specific against the HIV Gag SLYNTVATL (SL9) and the CMV pp65 NVLPMVATV (NV9) epitope. According to our previous unpublished observations and those results obtained in **paper IV**, we generally do not distinguish any differences of marker expression between MHC class-I tetramer stainings and short-term (<12 hours) stimulations.

3.3.2 Enzyme-Linked Immunospot (ELISPOT) assay

In order to screen for whether a specific peptide generates an antigen-specific response, the enzyme-linked immunospot (ELISPOT) assay is a perfect method. We used ELISPOT in **paper II**, to screen for a large number of peptides in many patients to distinguish HIV-specific T cell responses. ELISPOT is a very sensitive method that measures the frequency of cytokine secreting cells on single cell level. PBMCs are stimulated with peptides and then coated with antibodies on a culture surface. The antibodies will capture specific cytokines (IFNγ) that are secreted by the cells and after washing/removal of the cells, a substrate is added and a visible spot is distinguishable on the culture surface. This spot corresponds to a single IFNγ-secreting cell where the limit of detection could be as low as one cell in 100.000 (*212*).

ELISPOT has been in use for a long time due to its high sensitivity and ability to detect antigen specific responses with a low number of cells. However, the method has its disadvantages. As flow cytometry has established itself in most immunological laboratories, the method today possesses almost the same sensitivity and ability to detect antigen specific-responses as ELISPOT. Partly, this stems from flow cytometry's ability to detect multiple cytokines simultaneously, whereas traditional ELISPOT only enables single cytokine detection. In the case of T cell-specific responses, cell sorting would be necessary to delineate whether a positive response is of CD4+ or CD8+ T cell origin. This was apparent in **paper II**, where flow cytometry in the end was instead used to determine whether the peptide-specific responses were of CD4+ or CD8+ T cell origin.

3.3.3 Single-genome-sequencing (SGS)

Single-genome-sequencing (SGS) was developed to evaluate viral evolution and diversity of HIV from plasma (*213*). In **paper III**, HIV RNA was extracted from plasma and converted to cDNA by reverse transcriptase. Serial dilution of the cDNA allow for identification and sequencing of single HIV *gag* DNA molecules following polymerase chain reaction (PCR) amplification. In comparison with normal sequencing (population-based sequencing) measuring the dominant HIV species, SGS allows the detection of individual quasispecies of the virus.

3.4 BIOINFORMATICS

For all of these studies, bioinformatics were used to simplify the complex information

generated from both immunological and virological data. In addition, in **paper II** epitope selection algorithms were also used in order to pick out peptides that were d to generate MHC class-II restricted responses.

PICE

ed Presentation of Incredibly Complex Evaluations (SPICE) is an online software provided by Dr Mario Roederer at the National Institute of Health (*214*). The software is a data-mining programme that organizes highly complex polychromatic flow cytometry into potential graphical correlates in the data set. SPICE could be used for different aspects, where particularly polyfunctional characteristics of HIV-specific CD4+ (**Paper II**) and CD8+ T cells (**Paper III-IV**) have been used to detect multivariate functional correlates of disease progression. Specific phenotypic characteristics can also be assessed using SPICE, such as the identification of inhibitory receptors in **Paper IV** within defined T cell compartments (**Figure 7**). The software is generally used after applying combination (Boolean) gates to delineate multiple T cell populations in a given data set. SPICE essentially allows the determination of whether any differences exist between two or more groups based on these T cell populations using permutation and simple t-tests. However, although SPICE is an excellent tool for deciphering complex data sets, a larger number of populations are more difficult to analyze using the software.

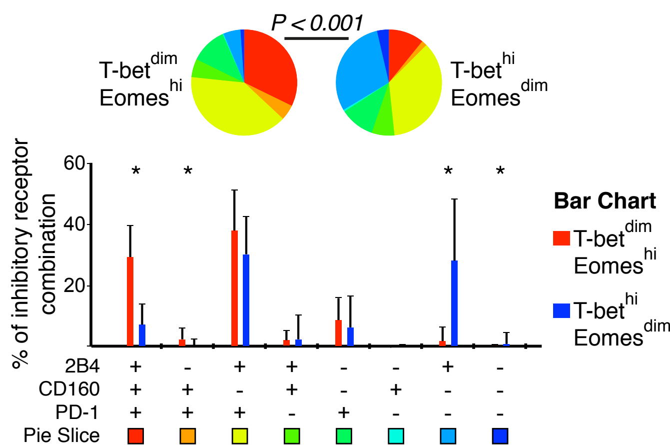


Figure 7. Illustration of SPICE diagram.

3.4.2 Principal component analysis (PCA)

In order to limit the vast amount of data that was generated from all T cell populations in **paper I** and **IV,** principle component analysis (PCA) was used. PCA is an unsupervised statistical method that reduces the dimensionality of data, while still retaining as much of the variation as possible in fewer variables. In **paper I**, Boolean gates were performed for all studied T cell markers and PCA was employed to reduce the dimensionalities and delineate differences between HIV-infected subjects versus healthy controls and HIV-infected individuals with low and high CD4/CD8 ratio. In **paper IV**, we combined all functional and inhibitory receptor characteristics with Boolean gates and used PCA to determine whether HIV- and CMV-specific CD8+ T cells had similar distributions.

3.4.3 Epitope selection

Many thousand different HIV strains have been characterized and, likewise, the restricting element of T cell responses consists of the highly variable MHC class I and II molecules, with more than 2000 allelic variants known. In former studies (*215, 216*), our group has used epitope selection algorithms to study CD8+ T cells responses directed against a broad set of HIV peptides. A similar approach was used in **paper II**, to select peptides generating broadly reactive MHC class II-restricted responses with respect to both the number of viral variants and MHC class II molecules targeted. A set of full-length HIV proteomes were scanned using NetMHCII (*217*) and NetMHCIIpan (*218, 219*) where we identified 15-mer peptides within the viral proteins Gag, Pol, Env, Nef, and Tat that were predicted to bind one or more of 45 prevalent HLA-DR and DQ alleles. From the peptide pool of more than 225,000 predicted binders, a final set of 64 peptides (15 Gag, 15 Pol, 15 Env, 15 Nef, and 4 Tat) was selected using a novel algorithm, PopCover, to ensure broad coverage of both the prevalent MHC class II alleles and the major HIV subtypes. These peptides were later shown to induce broad MHC class II-restricted responses in a heterogeneous cohort infected with different subtypes of HIV.

4. RESULTS AND DISCUSSION

HIV infects and kills CD4+ T cells that are the central players in normal homeostasis of effector immunity. The loss of CD4+ T cells generates a T cell repertoire that is highly dysfunctional and possibly therefore unable to clear the infection. Increased knowledge of impaired T cell dynamics will hopefully help develop our understanding of underlying mechanisms of HIV pathogenesis. In this thesis work, I have focused on integrating immunology and bioinformatics to study the complexity of T cell dysfunction in HIV pathogenesis. In the future, similar approaches will hopefully be rationales in *e.g.* vaccine development, primarily with the introduction of new techniques measuring multiple parameters simultaneously within the field of immunology (*220*).

Paper I: HIV infection is characterized by numerous pathological changes of the immune system. The immunopathogenesis is multifaceted, but most studies tend to focus on specific pathological markers (*e.g.* CD38 and HLA-DR) and rarely on the combination of numerous traits together. However, with the introduction of multi-parametric flow cytometry and advanced bioinformatics it has now become possible to simultaneously study these markers in the context of HIV induced T cell pathogenesis. Importantly, the immunopathogenesis, and primarily immune activation, is highly associated with disease progression to AIDS. Despite this notation, the CD4 count and VL still remain the routine laboratory parameters most commonly used to discriminate the state of HIV disease in the clinics today. Already in 1989, Taylor *et al* had distinguished that CD4/CD8 ratio and CD4% are slightly better disease progression predictors compared to CD4 count (*42*). Today it is widely known that in many clinical cases, despite fully recovered CD4 count after long-term ART, the CD4/CD8 ratio rarely reaches normal levels. In addition, recent studies have shown that the persistent lower CD4/CD8 ratio is a better correlate than CD4 count of sustained immune activation, exhaustion and senescence in HIV-infected individuals on ART (*43, 44*).

In this paper, we therefore conducted an interdisciplinary investigation, where multi-parametric flow cytometry and advanced bioinformatics were combined to determine which routine laboratory parameters are strongest associated with combined pathological changes of T cells. Markers of CD4+ and CD8+ T cell activation (CD38, HLA-DR), exhaustion (PD-1, Tim-3), senescence (CD28, CD57) and memory differentiation (CD45RO, CD27) were assessed retrospectively in a cohort of 47 untreated HIV infected individuals and 21 healthy controls. By combining all markers (using Boolean gating strategies) into 256 distinct CD4+ and CD8+ T cell populations respectively, we could distinguish a clear difference between the HIV-infected and healthy control subjects in terms of PCA and heat map analysis. Applying bioinformatical methods, we identified 139 distinct immunopathological populations, primarily expressing CD38, HLA-DR and PD-1, which significantly differed between HIV positive individuals and healthy controls (**Figure 8**).

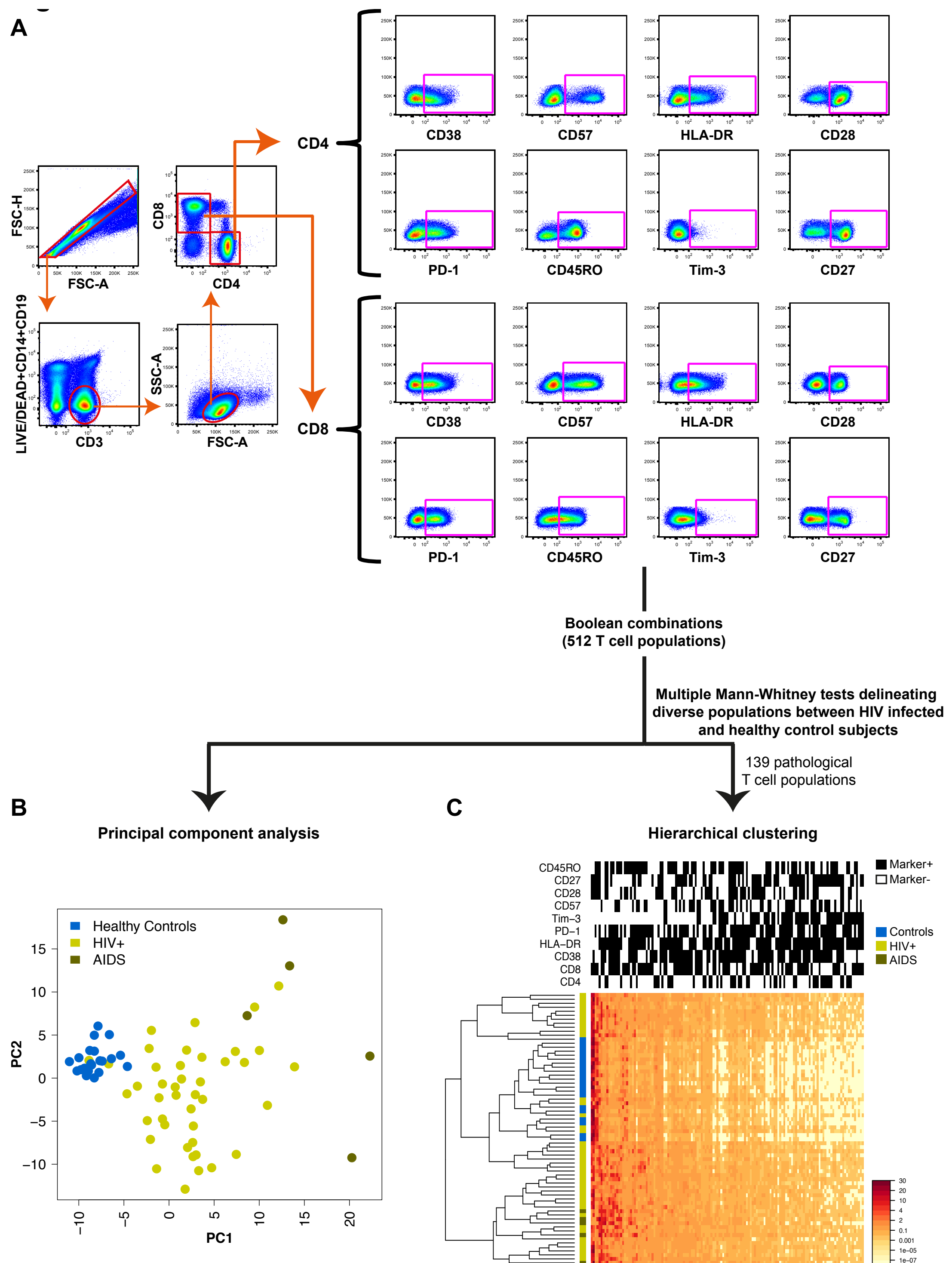


Figure 8. Schematic illustration of the pathways towards identification of combined pathological T cell populations.

We next applied the markers of T cell pathology and all 139 “pathological T cell populations” into stringent (Bonferroni adjusted) correlation analysis with all measured laboratory parameters (CD4 count, CD4%, CD8 count, CD8%, VL and CD4/CD8 ratio) in untreated HIV infection. It became particularly evident that the CD4/CD8 ratio was the preeminent surrogate of combined T cell pathology. The CD4/CD8 ratio was correlated to more of these markers and immunopathological populations (n = 10) than any other laboratory parameter. Interestingly, the ratio also had a significantly higher

average correlation coefficient compared to all other laboratory parameters, including the CD4%. Using advanced bioinformatics, Z-score transformations and PCA, to reduce the dimensionality of all immunopathological populations further identified the CD4/CD8 ratio as a better surrogate of combined immunopathogenesis than CD4 count.

In order to investigate which of the laboratory parameters that might have an impact on treatment outcome, we plotted the CD4 recovery 2 years post ART initiation and correlated this with baseline values of all markers of T cell pathology and laboratory parameters. In conjunction with the previous results, these analyses confirmed that the CD4/CD8 ratio, but not CD4 count, was associated with absolute CD4 recovery post ART. Likewise, the Z-scores and PC scores were also associated with the CD4 outcome post ART, which further indicate the relevance of measuring combined parameters of T cell pathogenesis and their influence on immune recovery. All of these analyses together concluded that the CD4/CD8 ratio (and CD4%) are strong surrogates of combined T cell pathogenesis that might be of interest in terms of ART initiation; particularly in resource limited settings where not all individuals might receive ART.

Paper II: The induction of HIV-specific CD4+ T cell responses in HIV infection and future vaccine trials remains controversial, particularly as these cells serve as a preferential site of HIV replication. Therefore, whether the presence of these cells is a consequence or cause of HIV disease progression or viral load remains hard to concretely determine. Nevertheless, recent studies have demonstrated that the breadth of Gag-specific CD4+ T cells is associated with lower viral load (*156*). Particularly, an inverted frequency of Env-/Gag-specific CD4+ T cell ratio seems correlated with the viral load, suggesting that targeting of conserved proteins by CD4+ T cells might establish pressure on the founder virus. However, a major challenge for the identification of HIV-specific CD4+ T cells targeting broadly reactive epitopes in populations with diverse ethnic backgrounds stems from the vast genomic variation of HIV and the diversity of the host cellular immune system.

In this study we therefore aimed to resolve the challenge of detecting broad HIV-specific CD4+ T cell responses. We developed an algorithm, called PopCover, that takes into consideration both optimal viral and host coverage. PopCover identified a set of potential HLA class II-restricted epitopes (n = 64), whereof a majority (73%) induced HIV-specific CD4+ T cell responses. Almost all Gag and Nef peptides induced responses and most of the responses were bound through predicted HLA-DR or –DP molecules. In correlation analysis, where only subjects with detectable viral load were included, the number of targeted Gag peptides was inversely correlated with viral load. Furthermore, we characterized the polyfunctionality of the responses against optimal Gag and Nef peptides in comparison with overlapping HIV-Gag p55 peptides. We found that the predicted peptides induced improved polyfunctionality compared to overlapping peptides, which further demonstrates that these peptides induce responses of high quality.

The concept of using bioinformatics and selection algorithms to generate broad T cell responses has previously been used in mosaic vaccine antigen trials. However, though

the mosaic protein sequence covers most viral strains, it ignores the aspects of generating responses of broad HLA diversity (*221*). In this paper we demonstrated that PopCover was able to resolve this dilemma and induced broader CD4+ T cell responses than previously described for comprehensive mapping of the HIV genome. All together, selection strategies such as PopCover might be used with success for the evaluation of antigen-specific CD4+ T cell responses and design of future vaccines.

Paper III: The rate of disease progression has been studied extensively in the context of untreated HIV infection. Although most individuals progress to AIDS within 10 years after infection, approximately 5-15 % remain immunologically stable despite lack of ART. This group of individuals have been named LTNPs, and a large proportion of them possess the HLA class I allele B-*5701 (*222*). Despite the fact that this allele has been linked to control of HIV viremia in numerous genome-wide association studies, only a small percentage of these subjects do progress at a slower rate (*i.e.* develop into LTNPs or elite controllers). Thus, how and why this allele is involved in host protection has remained unknown, but potentially involves both virological and immunological mechanisms.

In this paper we therefore wanted to determine for the first time, as far as we are aware, the virological and immunological factors linked to the risk of HIV disease progression in HLA-B*5701+ patients longitudinally. HIV *in vivo* evolution and wild type/autologous epitope-specific CD8+ T cell responses were studied in six untreated HLA-B*5701+ patients, monitored from early infection for up to 7 years. Individuals included in this study were categorized as high-risk progressors (HRPs) or low-risk progressors (LRPs) based on their baseline CD4+ T cell counts. Dynamics of HIV Gag p24 evolution was evaluated by high-resolution phylogenetic and multifunctional (IFNγ, TNF, IL-2 and perforin) CD8+ T cell responses were analyzed using polychromatic flow cytometry.

Based on the SGS data of HIV Gag p24 over time, we found that substitutions occurred more frequently in flanking regions than in HLA-B*5701-restricted epitopes. HRPs showed significantly higher Gag sequence diversity, lower homoplasy and less constrained mutational patterns compared to LRPs. In addition, HRPs had higher intrahost evolutionary rate and followed a specific molecular clock, which suggests that genetic drift rather than positive selection drives these events. Based on the immunological analysis, we distinguished decreased polyfunctional characteristics, primarily against the wild type TW10 and QW9 epitopes, for most time-points in HRPs compared to LRPs. These differences were primarily driven by IL-2 production, which was the main functional marker differing between the groups. The frequency of epitope-specific IL-2 producing cells was significantly associated with disease progression (CD4 count) longitudinally, but not viral load. These results are supported by unpublished observations (Buggert *et al*, Manuscript) where we have distinguished that the magnitude and IL-2 production *ex vivo* is higher for LRPs compared to HRPs against autologous and emerging HLA-B*5701-restricted epitope variants. Importantly, increased IL-2 production was highly associated with increased proliferation and perforin up-regulation after 3-day expansions.

In this study we demonstrated that interdisciplinary approaches combining advanced

virological and immunological methods through bioinformatics could identify risk factors linked to the rate of disease progression in HLA-B*5701+ subjects. The unpublished observations also implicate that IL-2 production facilitate perforin up-regulation after cell proliferation and might therefore determine the risk of disease progression in HLA-B*5701+ subjects. These studies highlight the importance of integrating multidisciplinary approaches to increase our knowledge of HIV disease progression in diverse individuals.

Paper IV: The repertoire of CD8+ T cells becomes highly dysfunctional after chronic exposure to viral antigens. This process is usually known as CD8+ T cell exhaustion and has previously been linked to the co-expression of several inhibitory receptors, including PD-1, CD160 and 2B4 in both mice and humans (*180, 181*). HIV-specific CD8+ T cells have particularly been studied in terms of dysfunctional characteristics, where seminal work has concluded that most individuals possess poor polyfunctionality (*174, 175*) and intermediate maturation phenotypes (*182, 183*). However, it remains unknown whether a transcriptional link exists between the regulation of CD8+ T cell differentiation and exhaustion in humans and HIV infection. Murine studies have clarified that the process of memory formation is highly regulated by the T-box transcription factors T-bet and Eomes. In recent reports from Wherry and colleagues, the exhausted profile following chronic viral infections in mice was associated with an inverse relationship between T-bet and Eomes (*116, 223*). Surprisingly, these studies showed that although T-bet caused terminal differentiation of CD8+ T cells, the transcription factor also bind directly to the promoter region of PD-1 and thereby inhibit the expression of inhibitory receptors. Eomes on the other hand was highly associated with expression of numerous inhibitory receptors. Despite these extensive studies in the murine model, surprisingly little is known about the influence of T-bet and Eomes on human CD8+ T cell exhaustion during chronic viral infections like HIV.

In order to shed light on this, we examined whether there is a link between the expression of T-box transcription factors (T-bet and Eomes) and markers of memory differentiation (CD45RO, CD27, CCR7), inhibitory receptors (PD-1, CD160, 2B4) and functionality (IFNγ, TNF, IL-2, CD107a and Granzyme B) in human bulk and virus-specific CD8+ T cells. In total, 52 individuals with chronic untreated HIV infection, 12 HIV infected individuals on ART for more than 10 years and 20 healthy controls were enrolled in this study. Out of the 52 individuals with chronic untreated HIV infection, 24 individuals were followed longitudinally from baseline and closely for 5-7 months post-ART initiation.

In this study we showed that PD-1, CD160 and 2B4 were highly elevated in untreated HIV+ subjects both on bulk and HIV-specific CD8+ T cells in comparison with healthy controls and CMV-specific CD8+ T cells. Increased expression of the inhibitory receptors was strongly associated with an inverse relationship between T-bet and Eomes. These cells, with a T-bet^{dim}Eomes^{hi} transcriptional profile, did not show features of terminal differentiation, but rather an intermediate (transitional) memory phenotype like previously annotated for HIV-specific T cells. Increased expression of Eomes in virus-specific CD8+ T cells was associated with single CD107a up-regulation and poor polyfunctional characteristics. Strikingly, the immature phenotype and

exhausted profile of HIV-specific CD8+ T cells remained stable after ART initiation and were accompanied by elevated levels of Eomes longitudinally, despite over 10 years on therapy.

This data provides a framework for why HIV-specific CD8+ T cells are potentially highly dysfunctional in chronic untreated HIV infection, and how future vaccines may need to overcome this transcriptional barrier and induce sustained T-bet expression in order to kill virus infected cells.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

Studies of T cell dynamics have been vital in understanding why AIDS development occurs at different rates after HIV infection. In this thesis, multidisciplinary approaches have been conducted to increase our knowledge of T cell pathology and dysfunction in HIV infection. The results described in these studies might be of interest and importance in future therapeutic and vaccine settings.

In all studies, combined approaches integrating immunology and bioinformatics were used to decipher the complex interplay between HIV and T cells. In **paper I** we used advanced bioinformatics and immunology to combine markers of T cell pathology with parameters of disease progression. Similar measurements were conducted in **paper IV**, but also using SPICE in **paper II-III**. Using this approach, we defined the CD4/CD8 ratio as a preeminent surrogate of T cell pathology and thereby confirmed similar observations to other studies of ART-treated subjects. In theory, our results might be of great interest in the context of ART initiation, since individuals with increased T cell activation have an increased risk of poor CD4 recovery (*80, 81*) and, also of non-AIDS-related morbidities (*224*). We largely confirmed these observations, and found that the state of combined T cell pathology is predictive of whether subjects initiating ART will have a good CD4 recovery or not. In addition, low CD4/CD8 ratios can exist in the presence of moderate CD4 count, and such individuals might therefore be at an increased risk for immunological failure. This observation was defined in our study, where several individuals with moderate CD4 levels (but low CD4/CD8 ratio) had poor CD4 recovery 2 years post ART initiation. However, this follow-up analysis was based on a limited number of patients, and therefore future cohorts of increased size should assess the link between CD4/CD8 ratios, T cell pathology, and the risk of immune recovery and non-AIDS morbidities after ART initiation. Other limitations in our data set include the lack of inflammatory markers (including TNF, IL-6), markers of microbial translocation (LPS etc) and other variables up-regulated in HIV infection that might impede CD4 recovery. However, our group has assessed several of these markers previously, and shown fluctuations that generally do not correlate with parameters of disease progression (like the CD4/CD8 ratio or CD4 count). Perhaps the most critical question of whether it is necessary to study markers of disease progression in untreated HIV infection is related to treatment guidelines. Today, individuals are generally treated before the CD4 count drop below 350 cells/μL, but future guidelines might insinuate introduction of ART in all phases of infection independently of CD4 count. However, in addition to the general interest of studying markers of disease progression from a “basic scientific point-of-view”, individuals in specific parts of the world might not be given ART at any phase of the infection in a near future. The main reason for this statement is the great cost of ART introduction for a longer period of time. For these individuals, measurements of disease progression (with *e.g.* CD4/CD8 ratio or CD4%) might be crucial before their immune system is too weak in order to recover post ART. Therefore, monitoring immunological parameters will hopefully be of significant importance in the future as well.

A general problem with the current T cell based vaccine trials stems from their abilities to generate broad T cell responses (*221, 225*). In **paper II**, we therefore developed an algorithm that resolves this challenge and identified peptides that were restricted to multiple HLA-DR and HLA-DQ alleles. We identified several novel peptides generating CD4+ T cell responses and confirmed a similar inverse correlation as Ranasinghe *et al*, between the number of targeted Gag epitopes and viral load. However, we noted that the subject with the highest number of targeted Gag peptides was an individual under ART with poor treatment adherence and blipping viremia. Thus, whether broad Gag-specific responses are true determinants of lower viremia remains uncertain in this study. HIV-specific CD4+ T cells are primarily infected with the virus, why increased antigen levels (due to high virus replication) may result in a directed depletion of these cells. Low levels of viral replication (not undetectable) might instead remain sufficient to prime and maintain functional CD4+ responses at higher quantities against HIV, without getting depleted. This idea is partly supported by data from other studies, which have discovered that HIV-specific CD4+ T cell responses are enriched in those with low-grade viremia due to treatment failure and natural HIV controllers (*147*). Nevertheless, Streeck and colleagues recently correlated specific HLA-DR alleles with lower viral replication in a large cohort of HIV infected subjects. These HLA-DR alleles were shown to possess lower functional avidity, but cross-presented numerous peptides for CD4+ T cells (*129*). In the STEP trial, the ability to induce broader T cell responses was actually linked to a lower viral load (*221, 225*). Similarly, recent studies from Picker and colleagues have demonstrated an extreme broadness of T cell responses induced by their CMV-vectors that might mediate the clearance of SIV (*159*). In this latter case, two-thirds of the CD8+ T cell responses were surprisingly shown to be MHC class II-restricted which further emphasizes the increased promiscuity of these molecules to present a large number of peptides. Particularly the mosaic vaccine antigens have tried to resolve the issue of generating broad T cell responses against different HIV strains (*214*) (these vaccines are under evaluation in clinical trials currently). However, although it might be reasonable to generate a broad antigen-specific T cell response, it should also be persistent over time. This was the general problem for example in the RV144 trial, where a clear demonstration of protective efficacy was distinguished in the first 6-12 months after vaccination, which vanished over time (*189*). In fact, no protection was demonstrated after this period of time, further emphasizing the importance of generating sustained B and T cell responses in future vaccine trials.

To date, a considerable number of studies have examined the events of HIV disease progression in HLA-B*5701+ patients (reviewed in (*226*)). We provide evidence in **paper III** that the risk of disease progression within these subjects is both associated to constrained evolution of HIV and polyfunctional characteristics of wild type/autologous CD8+ T cell responses longitudinally. Another consequence of viral control in HLA-B*5701+ subjects could otherwise stem from the composition of the TCR repertoire in specific individuals. Previous studies have linked public clonotypes to the development of MHC I-restricted escape (*227, 228*) and elite control (*136*). However, neither TCR diversity nor clonality of CD8+ T cells was recently demonstrated to differ between HLA-B*5701+ progressors and LTNPs (*140, 141*). Nevertheless, in terms of the functional characteristics, IL-2 in particular stood out as a correlate of decreased risk of disease progression early after infection in our study.

For most correlation analysis, though, it remains important to keep in mind what the causes and consequences are of disease progression. IL-2 secretion has previously been distinguished to increase post ART initiation, particularly for CD4+ T cells, which implicates that viral control leads to increased IL-2 expression and not vice versa (*153, 154*). However, in further studies we have distinguished that IL-2 secretion *ex vivo* is highly correlated with cell proliferation and perforin expression after 3-day expansions using optimal HLA-B*5701 restricted peptides (Buggert *et al*, manuscript). Thus, although different cell types might induce cytolytic and non-cytolytic characteristics, there is a clear link between these features, which indirectly has been demonstrated elsewhere (*105*). Whether the elicitation of IL-2 producing cells is of central importance in vaccine concepts remains to be seen, as previous studies have instead distinguished correlates of SIV disease protection with an effector memory response (producing high levels of IFNγ, TNF, CD107a and MIP-1β) (*202, 203, 229, 230*). IL-2 secretion is primarily distinguished in central memory cells, which are less mature and often re-circulate between the lymph nodes and blood. In order to elicit a protective response at sites of infection like the mucosa, CD8+ T cells probably need to reside at high quantities in these tissues and intercept the virus before it reaches the lymph nodes and disseminates to the rest of the body. Whether this is possible to achieve with IL-2 secreting cells or central memory cells is also, as yet, unclear.

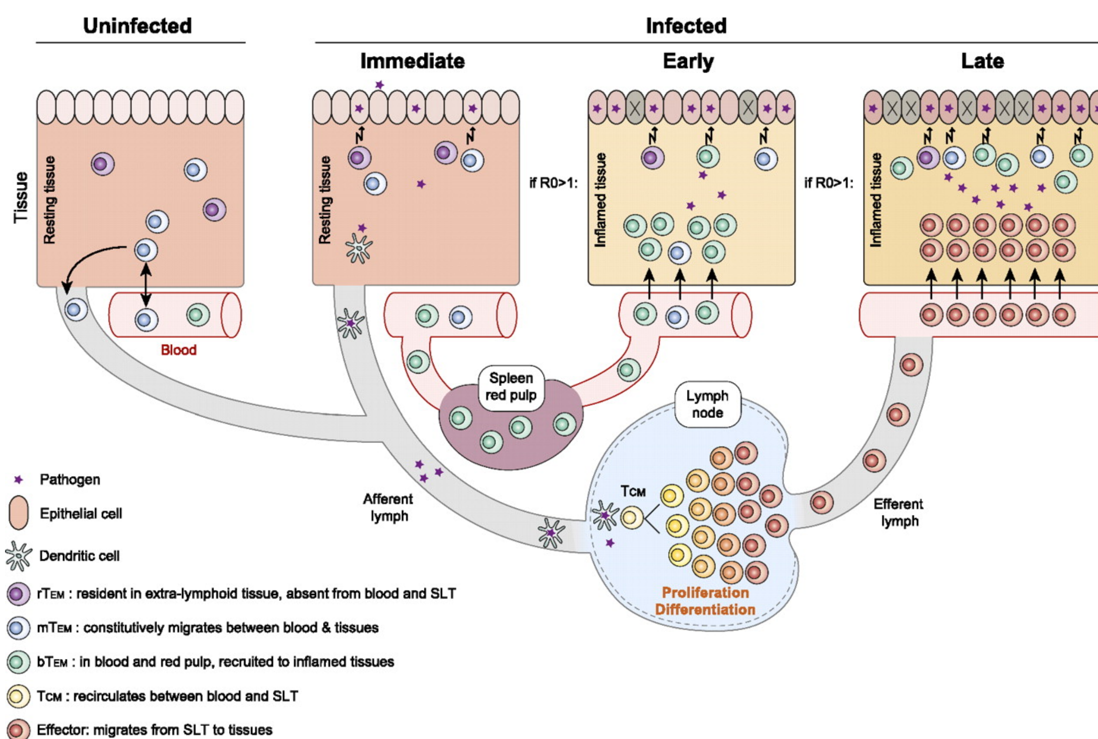


Figure 9. The contribution of diverse memory T cells varies after pathogen invasion and different subsets mediates an immediate or delayed response depending on their localization within the body. Reprinted with permission from (*231*).

A general issue in chronic HIV infection is the lack of functional CD8+ T cell clones to combat the infection. The process of CD8+ T cell exhaustion is thought to be a consequence of increased inflammation, antigen load, and other events that drive the

cells to an end-stage of their life cycle where they lose the ability to proliferate and induce effector functions (*176*). This dogma is supported by the fact that high antigen levels have been shown to cause T cell exhaustion during chronic viral infections in mice models (*232*). In addition, HIV+ elite controllers generally have higher T cell polyfunctionality (*175*) and lower expression of inhibitory receptors on CD4+ T cells (*233*). However, not all elite controllers show low expression of inhibitory receptors (unpublished observations) and despite viral control by ART, functional characteristics of HIV-specific CD8+ T cells are not fully restored (*175, 234, 235*). Whether the state of exhaustion is directly proportional to antigen burden and due to chronicity is not entirely clear. In **paper IV** we tried to conduct a multi-parametric study to link several features of CD8+ T cell dysfunction with the expression of certain T-box transcription factors (T-bet and Eomes). Previous studies in mice have shown that CD8+ T cells with exhausted phenotypes have elevated levels of Eomes but low T-bet expression (*116*). These results conflicted with those previously published by another group, which suggested that mRNA levels of T-bet and Eomes are both down-regulated in HIV-specific CD8+ T cells (*236*). In this thesis, I have developed techniques to detect protein expression of transcription factors directly with flow cytometry on single cell level, while the mRNA levels of these transcription factors might be difficult to detect using PCR techniques in resting cells where multiple genes are measured simultaneously.

In our study we corroborated the findings distinguished in mice that T-bet and Eomes are differentially linked to CD8+ T cell exhaustion also in humans. The inverse relationship between T-bet and Eomes in HIV-specific CD8+ T cell has been detected by other collaborators (unpublished observations) and confirmed using immunoblot techniques (*237*). Probably the most surprising results in our study (**paper IV**) was the fact that residual HIV-specific CD8+ T cells after long-term therapy still showed extensive signs of exhaustion, which was linked to persistent levels of Eomes. The persistent exhausted phenotype of virus-specific CD8+ T cells has also been observed in mice after antigen withdrawal (*238*) and might be a consequence of unmethylated promotor regions of inhibitory pathways (*239*). The imprinted phenotype of T-bet and Eomes in HIV-specific CD8+ T cells implicate that ART does not change many other parameters as well in addition to those studied by us. The sustained expression of Eomes in HIV-specific CD8+ T cells after long-term ART further suggest that therapeutic strategies aiming at reinvigorating these responses might fail to elicit efficient responses to eradicate the viral reservoir. Therefore, future vaccine or cure approaches most probably need to elicit new CD8+ T cell clones or find ways to overcome the T-bet^{dim}Eomes^{hi} transcriptional barrier in order to clear or kill virus-infected cells.

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