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IMMUNE DETERIORATION IN HIV-1 AND HIV-2 INFECTION WITH A FOCUS ON CD8 T CELL EXHAUSTION

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Immune deterioration in HIV-1 and HIV-2 infection with a focus on CD8 T cell exhaustion
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By

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To my loved ones.

ABSTRACT

Thanks to the development of antiretroviral treatment (ART), human immunodeficiency virus (HIV) infection is now considered a chronic infection rather than the death sentence it used to be. Despite constant expansion and improvement of treatment options, even optimal therapy cannot prevent the impact of HIV on the human immune system. Persistence of latently infected cells, low-level residual viral replication and long-term impact from initial tissue damage lead to chronic immune activation, which in turn drives a number of comorbidities and complications that lower the patient´s quality of life.

The hallmark of HIV infection is the depletion of CD4 T cells, also called helper T cells. In addition to immune activation due to the virus itself, the lack of support by CD4 T cells impairs numerous immune cell types. As the main defender against viral infections, CD8 T cells play a central role in ending the peak of viral replication during acute HIV infection. However, during the subsequent chronic phase, they become exhausted, which impairs not only immune responses against HIV, but also other viral infections and malignant cells.

In **paper I** we investigate the role of the immune receptor TIGIT in HIV type 1 (HIV-1) infection. As an exhaustion marker, it is increased in HIV-1 infection, co-expressed with other inhibitory receptors and fails to return to normal levels despite viral suppression by ART. It is also part of a regulation network together with the complementary co-stimulator CD226 and their shared ligand PVR. All members of this network are skewed in HIV-1 infection and contribute to CD8 T cell exhaustion and immune deterioration.

Of the few human genetic factors beneficial in HIV-1 infection, HLA-B*57 has the potential to slow down disease progression. **Paper II** investigates the connection between HLA-B*57 and exhaustion of HIV-specific CD8 T cells. Delayed co-expression of TIGIT and PD-1 might be one factor contributing to slower disease progression often seen in people positive for the HLA-B*57 allele.

HIV type 2 (HIV-2) is associated with slower disease progression and a higher chance of virus control by the host immune system than HIV-1. In **paper III**, we found signs of CD8 T cell pathogenesis, especially a skewed balance of co-stimulation and inhibition, in HIV-2 infection despite controlled viral replication.

HIV-1 pathogenesis in children after mother-to-child transmission shows differences from pathogenesis in adult patients and leads to unique complications. To better understand the unique aspects of pediatric HIV infection and potentially find candidates for therapeutic intervention, we investigated plasma biomarkers in well treated children in **paper IV**. Their treatment prevents high levels of dysregulation in comparison to HIV-negative children. However increased levels of sTRAIL, a marker for T cell activation with potential to induce apoptosis, shows the capacity to further optimize treatment in HIV infected children.

LIST OF SCIENTIFIC PAPERS

I. Perturbed CD8+ T cell TIGIT/CD226/PVR axis despite early initiation of antiretroviral treatment in HIV infected individuals; Tauriainen J, **Scharf L**, Frederiksen J, Naji A, Ljunggren H-G, Sönnerborg A, Lund O, Reyes-Terán G, Hecht FM, Deeks SG, Betts MR, Buggert M and Karlsson AC;
Scientific Reports 7, 40354 (2017)*

II. Delayed Expression of PD-1 and TIGIT on HIV-Specific CD8 T Cells in Untreated HLA-B*57:01 Individuals Followed from Early Infection; **Scharf L**, Tauriainen J, Buggert M, Hartogenesis W, Nolan DJ, Deeks SG, Salemi M, Hecht FM and Karlsson AC;
Journal of Virology 94:e02128-19 (2020)*

III. Inverted balance of CD8 T-cell exhaustion and co-stimulation markers differentiate aviremic HIV-2-infected from seronegative individuals; **Scharf L**, Pedersen CB, Lindman JL, Olsen LR, Buggert M, Wilhelmson S, Månsson F, Esbjörnsson J, Biague A, Medstrand P, Norrgren H, Karlsson AC, Jansson M, and the SWEGUB CORE Group; *manuscript*

IV. Plasma biomarkers identifying immunological reconstitution in perinatally HIV-infected children undergoing antiretroviral treatment; **Scharf L**, Olofsson A, Soeria-Atmadja S, Thalme A, Navér L, and Karlsson AC; *manuscript*

CONTENTS

LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
APOBEC3	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3
ART	Antiretroviral treatment
CAR T cell	Chimeric antigen receptor T cell
CCL3	C-C motif chemokine ligand 3
CCR5	C-C chemokine receptor type 5
CMV	Cytomegalovirus
CRF	Circulating recombinant form
CRISPR	Clustered regularly interspaced short palindromic repeats
CTLA-4	Cytotoxic T-lymphocyte associated protein-4
CXCR4	C-X-C motif chemokine receptor 4
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
dNTP	Deoxynucleoside triphosphates
EBV	Epstein-Barr virus
Env	HIV envelope glycoprotein
Eomes	Eomesodermin
FCM	Flow cytometry
GALT	Gut associated lymphoid tissue
GWAS	Genome-wide association studies
HAVCR2	Hepatitis A virus cellular receptor 2
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HLA	Human leukocyte antigen

IFNγ	Interferon-γ
IL-2	Interleukin 2
INT	HIV integrase
IP-10	Interferon-inducible protein 10
IRIS	Immune reconstitution inflammatory syndrome
ISG	Interferon stimulated gene
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITSM	Immunoreceptor tyrosine-based switch motif
IVDU	Intravenous drug user
KIR	Killer immunoglobulin-like receptors
KLRG1	Killer cell lectin like receptor G1
Lag-3	Lymphocyte activating 3
LPS	Lipopolysaccharide
LRA	Latency reversing agent
LTNP	Long-term non-progressor
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein 1α
mRNA	Messenger RNA
MSM	Men who have sex with men
MTCT	Mother-to-child transmission
NF-κB	Transcription factors like nuclear factor κB
NFAT	Nuclear factor of activated T cells
NK cell	Natural killer cell
NNRTI	Non-nucleoside reverse transcriptase inhibitors
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood monocytes
PD-1	Programmed cell death 1
PD-L1	PD-1 ligand 1
PLHIV	People living with HIV
PR	HIV protease
PRR	Pathogen recognition receptor

PVR	Poliovirus receptor
RANTES	Regulated upon activation, normal T cell expressed and secreted
RT	HIV reverse transcriptase
SAMHD1	SAM and HD domain containing deoxynucleoside triphosphate triphosphohydrolase 1
SIV	Simian immunodeficiency virus
SOMs	Self-organizing maps
SPICE	Simplified Presentation of Incredibly Complex Evaluations
T-bet	T-box transcription factor 21
TCR	T cell receptor
T_{FH}	T follicular helper cell
TGFβ	Transforming growth factor β
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TOX	Thymocyte selection associated high mobility group box
TRAIL	TNF-related apoptosis-inducing ligand
TRIM5α	Tripartite motif containing protein 5α
VL	Viral load
WHO	World health organization

1 BACKGROUND

1.1 THE HUMAN IMMUNODEFICIENCY VIRUS PANDEMIC

1.1.1 Epidemiology and current status

Awareness of acquired immunodeficiency syndrome (AIDS) first arose in the early 1980ies, starting with the report about five cases of pneumonia in previously healthy men who have sex with men (MSM) by physicians in Los Angeles (1). Following this, more cases of pneumonia, Kaposi´s Sarcoma and other opportunistic infections were reported from the MSM community, drug users and recipients of blood transfusions. Epidemiologists urged for surveillance of new cases, defined risk groups and revealed routes of transmission. Important research advances were the isolation of the causative agent, the human immunodeficiency virus (HIV), in 1983 (2), the development of diagnostic tests (3, 4) and cloning, as well as sequencing of the viral genome (5-7).

Analysis of viral genomes revealed that HIV was a zoonotic infection that crossed the species barrier between chimpanzees and humans (8, 9). Urbanization, colonization, increased transcontinental travelling, and other human behaviors allowed the virus to disseminate globally and lead to manifestation of the HIV/AIDS pandemic with increasing rates of HIV infections until 1998. Since then, the development of antiretroviral drugs targeting different steps in the HIV replication cycle, effective combination therapy, and global efforts to diagnose and treat HIV have reduced the rate of new infections. Simultaneously, the improved access to and quality of medical care prolonged the survival of people living with HIV (PLHIV) to almost normal life spans.

According to UNAIDS estimations, 38 million people lived with an HIV infection in 2019, 1.8 million of which are children younger than 15 years. 1.7 million were newly infected in 2019, 150 000 of which were children. Of the PLHIV that knew about their infection, 66.8% had access to antiretroviral treatment (ART) (10). This number is even lower among children, where only 53% of infected individuals received ART. 690 000 PLHIV died of AIDS-related causes in 2019. Despite great success, this is still considerably higher than the intended reduction below 500 000 deaths by 2020. Global institutions have expressed the goal to diagnose 90% of PLHIV, provide ART to 90% of diagnosed individuals and achieve viral suppression in 90% of treated PLHIV by 2020. This 90-90-90 target has been achieved in a number of countries, among which Sweden was the first by accomplishing the goal in 2015 (11).

1.1.2 Viral diversity and global distribution

HIV belongs to the genus *Lentivirus*, within the family of *Retroviridae*. Today, two types of HIV are known, HIV type 1 (HIV-1) and HIV-2. HIV-1 comprises four groups (M - major, O – outlier, N - non-M/non-O and P). The global HIV pandemic is caused by HIV-1 group M. HIV-2 is relatively confined to Western Africa and countries with social and economic

connections to this region. HIV-2 is considered a “mild” form of HIV due to slower disease progression and lower transmission rates.

One reason for the phylogenetic diversity of HIV is the zoonotic origin. In old world monkeys, more than 40 different simian immunodeficiency viruses (SIV) are known. SIV infections typically occur within the same monkey species and, as a result of co-evolution of virus and host, cause mild to no disease. HIV-2 is the result of eight transmissions from sooty mangabeys to humans (12), resulting in 8 lineages. In contrast, SIV of chimpanzees (SIVcpz) developed from two monkey SIV clades that recombined after transmission from their original hosts to chimpanzees. After transmission to humans, SIVcpz disseminated as HIV-1 group M and N. SIVcpz also crossed into gorillas, creating SIVgor and subsequently HIV-1 group P and O (12, 13). Since the dissemination of HIV-1 group M, it has diversified into several subtypes or clades (A, B, C, D, F, G, H, J and K) as well as circulating recombinant forms (CRFs). The dissemination of these subtypes differs vastly between the continents. Subtype B is the most prevalent virus in North America, Australia, the majority of Europe and parts of South America. Subtype C is prevalent in the South-East of Africa and India, while other parts of the world, especially sub-Saharan Africa, show a more complex distribution of two or more subtypes and CRFs (14).

1.1.3 Transmission routes of HIV

HIV transmissions can occur via sexual contact, parenteral via blood or cerebral spinal fluid, and through mother-to-child transmission (MTCT) during pregnancy, birth or breastfeeding. Depending on the type of exposure, the transmission risk can vary from 0.08% per exposure from insertive heterosexual intercourse (15) up to more than 90% from transfusion of infected blood (16).

While the original transmission between monkeys or apes to humans is believed to be the result of contact with infected blood when hunting the animals, the dissemination of HIV-1 group M was enabled by migration from rural areas to Kinshasa (17). Heterosexual transmission, in particular through sex workers, as well as the use of unsterile needles resulted in fast dissemination of the virus. From Kinshasa, the virus spread to the Caribbean and subsequently to North America, where it was already present in the 1970s (18), but went undetected for another decade. As the resulting disease, AIDS, was first discovered in MSM in the early 1980s, it was initially associated with homosexual transmission. However, use of unsterile needles among intravenous drug users (IVDU) and use of blood products in health care interventions substantially contributed to establishment of the pandemic. While screening of blood donations and improvement of hygiene practices has virtually eliminated the risk of HIV infection via health care systems, there is still a large difference in the mode of HIV transmission between different parts of the world. In Western and Central Europe as well as North America 64% of HIV transmissions are related to MSM. Similarly, this is the most common transmission route in Latin America, Asia and the Pacific. However, in these regions, 25 - 30% of new infections are related to either sex work or partners of people at high risk. In the Middle East, North Africa, eastern Europe and central Asia, people who

inject drugs are at the highest risk of HIV infections. This is a stark contrast to eastern and southern Africa, where only 28% of all HIV transmissions can be attributed to risk groups.

In order to reduce transmission rates, numerous organizations offer programs ranging from large scale awareness campaigns for the general public to national efforts such as needle exchange programs for IVDUs. One area of great success is the prevention of MTCT. HIV testing of pregnant women and administration of ART to infected mothers during pregnancy and breastfeeding have radically decreased the number of MTCTs globally and more specifically in Africa (19). The remaining MTCTs are often linked to particularly vulnerable groups, such as young women with little access to health care systems, insufficient education, experiencing violence and stigma, or similar social and economic problems (10). Similarly, HIV infected children not receiving ART often belong to particularly vulnerable populations, such as orphans of HIV infected parents, or were never tested for HIV despite having infected family members.

1.2 NATURAL COURSE OF HIV INFECTION

1.2.1 Transmission and acute infection

HIV particles can be detected in blood, semen, pre-seminal, vaginal and rectal fluid as well as breast milk (20). Via abrasions or lesions in mucosa or skin, HIV can enter submucosal tissue and is typically taken up by dendritic cells (DCs) as they patrol the tissue (21). DCs then transport the virus to lymphoid tissue, where large quantities of CD4 helper T cells are present. As these CD4 T cells are the main target cells for HIV infection, lymph nodes are a major site for HIV establishment, spread, and persistence. Alternatively, CD4 T cells can be infected at the site of HIV entry, if they are present either as tissue resident cells, have recently entered the already inflamed site, or if HIV contaminated fluids come into direct contact with circulating blood. Inflamed lesions due to other pathogens facilitate HIV infection by destruction of the mucosal barrier and recruitment and activation of immune cells. This biological mechanism explains why other sexually transmitted diseases are often risk factors for HIV infection (22).

As an enveloped virus, HIV is surrounded by the cell membrane from an infected host cell, which contains the viral envelope glycoprotein (Env). Upon contact with the target cell, Env consecutively binds to two receptors, the main receptor CD4 (23, 24) and a co-receptor (25-28). CD4 is expressed by T cells and monocytes/macrophages, and binding to CD4 on the target cell leads to conformational changes of Env, bringing the virion closer to the cell. This change also exposes the binding site for the co-receptor, which in the majority of HIV transmissions is C-C chemokine receptor type 5 (CCR5). Alternatively, HIV can mutate to bind to C-X-C motif chemokine receptor 4 (CXCR4) as a co-receptor, which is usually associated with later disease stages (28). The particles can thus be classified into R5- or X4-tropic virus. In contrast to HIV-1, HIV-2 can bind to a multitude of co-receptors (29). Binding to the co-receptor initiates membrane fusion and release of the inner components of HIV virions into the cytoplasm. HIV particles contain a conical core of capsid protein

encasing two positive sense, single-stranded RNA molecules and three viral proteins: reverse transcriptase (RT), integrase (INT) and protease (PR). RT synthesizes complementary DNA from the RNA template, the step in their life cycle that retroviruses are named for. The enzyme lacks proof-reading ability, resulting in a high rate of mutations per replication. INT subsequently incorporates the viral DNA into the human genome. For synthesis of messenger RNA (mRNA) and viral proteins, the virus hijacks the host cell transcriptional and translational machinery. HIV, as many other viruses, minimizes it´s genome size by using several open reading frames and encoding polyproteins, that then need to be cleaved by PR or a cellular protease into their functional protein subunits. From the structural proteins and RNA, HIV virions assemble and, after budding off their cell of origin, infect their next target cell. In addition to the building blocks for new virions, *i.e.* the structural proteins, HIV also possesses a number of accessory proteins, some of which interact with host proteins to evade the immune system, others regulate viral gene expression.

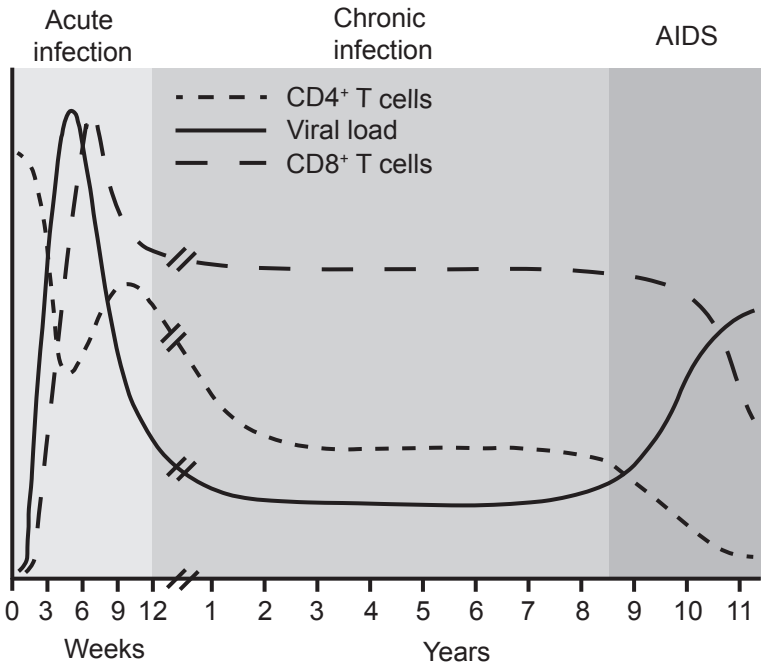

Figure 1. Progression of plasma viral load, CD4 and CD8 T cell counts in HIV infection. Re-printed with permission from (30).

As T cells enter circulation, HIV disseminates rapidly throughout the human body, especially lymphoid tissues. Consequently, a high number of cells are infected, resulting in a high virus concentration in blood, both as free virions (31), and as infected cells (32). As a quickly disseminating cytopathic virus, HIV causes a sudden depletion of its target cells, CD4 T cells (Fig. 1). A large proportion of human CD4 T cells resides in the intestinal lamina propria and gut associated lymphoid tissues (GALT) (33), creating an optimal hotspot for HIV replication and CD4 T cell depletion during peak viremia. CD4 T cells are not merely residing in the gut, but are integral to homeostasis and barrier function (34). The depletion of more than 70% of intestinal CD4 T cells, especially the subset of Th17 cells (35), during primary HIV infection thus damages the structures these cells reside in (36). This damage allows microbial

components like lipopolysaccharide (LPS) to reach the circulation, a process called microbial translocation (37) and a driving force behind activation and exhaustion of the cellular immune system. Similarly, both the thymus and lymph nodes, essential for renewal and maintenance of the CD4 T cell pool, suffer from structural destruction. Reduced thymic function impairs production of naïve T cells (38, 39) and thus contributes to loss of CD4 T cells beyond cytopathic effects (40). Insufficiently replacement of Th17 cells links loss of thymic function to microbial translocation.

On average 14 days after infection, the rapid increase of viral replication can cause symptoms typically associated with viral infections, such as fever, pharyngitis, rash, diarrhea, and general weakness. Approximately three weeks after symptom onset, the peak viral load and initial CD4 T cell depletion is in part controlled by the development of HIV-specific CD8 T cells (Fig. 1) (41). The importance of this cell type was first seen in *in vitro* experiments (42) and later confirmed using SIV-infected monkeys depleted of CD8 T cells (43). Without CD8 T cells, the immune system fails to end the increase in viral replication and infection of new target cells, accelerating mortality in the monkeys. Despite ending peak viremia, the immune response against HIV is not capable of completely eliminating the virus and HIV establishes a chronic infection.

Furthermore, B cells develop HIV-specific antibodies. B cells become highly activated and develop neutralizing antibodies against HIV in the vast majority of patients. However, HIV escapes antibody responses with escape mutations and by shielding HIV Env with glycans. Furthermore, HIV decreases the quality of antibody responses over time by exhausting and dysregulating B cell responses.

1.2.2 Chronic Infection

Already the first report of HIV in 1981 noted the deficiency of the cellular immune system seen in HIV infected individuals, which was soon connected to the depletion of CD4 T cells. Thus, two parameters, plasma viral load (VL; number of RNA copies per mm^3 plasma) and CD4 T cell count (number of CD4 T cells per mm^3 blood), became the standard way to monitor patients with regards to disease progression.

After the acute stage of HIV infection, CD4 T cells can recover partially (44), but over the course of the subsequent chronic phase, CD4 T cell depletion continues at a slow rate (Fig. 1). A few weeks to months after infection, the viral load reaches a more or less stable level, termed the viral set point. The viral set point is indicative of the rate of disease progression (45), which is highly variable between individuals. A very small group of patients, so-called elite controllers, can suppress viral replication below standard detection limits over several years. Another group, often called viremic controllers, suppress viral load below usual levels, but above detection limit. In contrast, long-term non-progressors (LTNPs) are defined by their maintenance of CD4 T cells regardless of viral load. Despite commonly used terms, the exact values (VL, CD4 T cell count, and duration of control/maintenance) used to define these categories can change between studies. However, the vast majority of untreated HIV

infections progress to AIDS within less than 10 years, and rapid progressors can experience AIDS within a year or less after infection (46).

During chronic infection, the persistence of viral antigen leads to immune activation and low inflammation, which in turn fuels immunosenescence (47). The replication capacity of the HIV founder virus (48), the virus that established HIV infection in a host, influences the severity of immune activation. Furthermore, co-infections contribute to the immune activation (49) and the damage to lymphoid tissues progresses during chronic infection and exacerbates immune dysregulation.

HIV causes CD4 T cell depletion not only directly through cytopathogenicity of infected cells, but also by cell death of bystander cells (50). A highly inflammatory type of cell death, pyroptosis, is common in HIV infection (51). As a result of pyroptosis, cytoplasmic content of the dying cell and cytokines are released, which serve as additional proinflammatory signals. In addition to cell death, regeneration of CD4 T cells is compromised. Thymic function, essential for T cell development, is impaired (38) and infection of progenitor cells (52) abrogates their potential to proliferate and thus maintain CD4 T cell numbers.

PLHIV typically do not experience symptoms during the chronic stage HIV infection. However, without ART initiation and adherence, HIV infection progresses to AIDS as CD4 T cell counts become insufficient to maintain host defense.

1.2.3 Antiretroviral therapy

In 1987, the first drug against HIV, azidothymidine (53), which is now called zidovudine, was licensed. As a nucleoside reverse transcriptase inhibitor (NRTI), it targets the viral reverse transcriptase and prevents synthesis of DNA from the viral RNA template. The drug resulted in a temporary reduction of viral replication, but drug resistances developed rapidly due to the high mutation rate and virion production of HIV (54). Further NRTIs were developed in the following years, but only with the first protease inhibitor (PI), approved as Saquinavir in 1995, was it possible to target a different step in the HIV life cycle. Major improvement in the form of durable suppression of viral replication was achieved by the introduction of combination therapy (55, 56), using two NRTIs together with one PI. Triple therapy has since been standard in the treatment of PLHIV. Over the years, non-nucleoside reverse transcriptase inhibitors (NNRTIs) as well as entry, fusion and integrase inhibitors expanded the repertoire of HIV drugs (57). Thus, through modern ART we can effectively inhibit viral entry, replication and maturation but not neutralize virions or eliminate latently infected cells.

In addition to HIV infection resulting in virus production by infected cells, it also leads to latent infection where HIV stays dormant inside long-lived memory T cells (58), myeloid cells and probably hematopoietic progenitor cells (59). Because these latently infected cells cannot be targeted by current ART, they make up the viral reservoir in treated PLHIV and spontaneously re-establish infection once ART is discontinued (60). Only a small proportion of patients that initiated ART very early in HIV infection can develop post-treatment control

(61), a phenomenon without clear definitions regarding the extent of viral load suppression or duration of control. It is unclear whether latent infection is established by infection of active CD4 T cells and escape from cell death when transitioning to a resting state (62), or whether HIV is indeed capable of infecting resting memory cells (63). However, sequence analysis of the viral reservoir revealed that it is established very early and continues to grow throughout the infection (64).

In addition to latently infected cells, HIV maintains low levels of viral replication even after years of viral suppression by ART (65). Incomplete drug penetrance into specific anatomical sites, *e.g.* lymph nodes or the central nervous system, allows viral replication and consequential *de novo* infection (66, 67).

Until 2015, treatment was initiated when CD4 T cell counts reached a certain level, which changed as new evidence was reported (*e.g.* 500 cells/mm^3 was the recommended threshold for initiation from 2013 to 2015). However, earlier treatment was linked to improved outcome, leading to the current recommendation to start treatment upon diagnosis, regardless of CD4 T cell count (68). Corresponding to the clinical monitoring of CD4 T cell counts and VL, two outcomes define successful ART: suppression of VL below detection limits and reconstitution of CD4 T cell counts. Virological failure is defined as failing to achieve or sustain VL below 200 copies/mL (69) and testing for resistance mutations typically guides medication change, though problems in adherence, drug absorption or interactions with other drugs can be alternative explanations (70). In contrast, there is no uniform definition for immunological failure and different thresholds of CD4 T cell count, % CD4 T cells (CD4%), or increase in CD4 T cell count above pre-treatment baseline are used (71). The cause for immunological failure is also not straightforward, usually including several factors impacting production or depletion of CD4 T cells. However, choice of the optimal ART regimen has the potential to aid immune recovery (72).

As seen for early monotherapy, HIV quickly develops drug resistance when given the opportunity to replicate under suboptimal concentrations of the drugs. Viral replication despite treatment due to problems in ART adherence or access, and inadequate treatment due to undiagnosed virological failure are the main reasons for acquired drug resistance. However, virus with established resistance mutations is commonly less fit than drug sensitive sequences and after discontinuing ART the proportion of circulating resistant virus declines (73). The increased access to ART under suboptimal conditions increases the occurrence of transmitted drug resistance (74) and as long as vaccines or a cure for HIV remain elusive, resistance will pose an increasing challenge in the fight against the HIV pandemic. In the meantime, the continued spread of HIV is addressed with strategies like pre-exposure prophylaxis (75), or development of antiretroviral microbicides administered as vaginal gels (76). Furthermore, adherence problems, and thus resistance, could be limited by administration of long acting ART (77).

Adverse effects due to ART toxicity include dermatologic manifestations such as rash, neurotoxicity, hepatotoxicity (78), renal disease and reduced bone density (79). Development

of modern drugs has decreased the prevalence of adverse effects, and screening for known risk factors, such as human leukocyte antigen (HLA) B*57 which is correlated to abacavir hypersensitivity (80, 81), can help to prevent them. However, ART toxicity is still a considerable contributor to comorbidities in PLHIV, especially as survival increases (82, 83).

A small group of PLHIV who start ART can experience immune reconstitution inflammatory syndrome (IRIS). Recovery of CD4 T cells upon initiation allows immune responses that were previously suppressed by HIV to rise (84). Thus, IRIS is more common in PLHIV with lower CD4 T cell counts prior to ART initiation (85, 86) and rapid recovery (85, 87). A possible explanation for IRIS is a difference in recovery rates between CD4 T cell subsets. A delay in regulatory T cell recovery in comparison to other subsets might leave immune responses unchecked until this delay is overcome (84). Prevention of IRIS is possible by screening PLHIV for opportunistic infections that are typically associated with IRIS, such as tuberculosis, other mycobacterial infections, Cryptococcus or viral infections. However, delaying ART initiation in order to avoid IRIS risks HIV disease progression. Furthermore, antigen associated with IRIS is not necessarily derived from active infections, but can also come from tumors, dead pathogens from resolved infections or even self-antigen, causing autoimmune symptoms (84).

1.2.4 Acquired immunodeficiency syndrome

Untreated HIV infection results in AIDS, when the immune deterioration renders host defense insufficient to maintain health. CD4 T cells are essential in orchestrating immune responses, development of B cell responses, maintenance of CD8 T cell memory, and functions of innate immune cells *e.g.* phagocytosis by macrophages. Through the lack of CD4 T cells, these other immune cell types cannot maintain or execute their functions. Latent infections, such as cytomegalovirus (CMV), tuberculosis or herpes viruses, are no longer contained and can now cause death of severely immunocompromised patients. Typical AIDS-related malignancies are Kaposi´s sarcoma and B cell lymphomas, which are themselves caused by viral infections that are no longer contained, such as human herpesvirus 8 and Epstein-Barr virus (EBV) (88). However, malignancies can also be the result of cytokine dysregulation (89). In addition to these AIDS-related conditions, a CD4 T cell count below 200 cells/mm^3 is a criterion for AIDS diagnosis. As with other infections, the partial containment of HIV seen in the chronic phase ends and viral replication increases again.

1.2.5 Pediatric disease progression

MTCT can occur late in pregnancy, at birth or during breastfeeding. The transmission risk from untreated mothers to children varies from 15 to 35% (90) and depends heavily on disease severity of the mother and the frequency of breastfeeding. Other factors that influence the risk of transmission are related to the immune system or of genetic nature. Similarities in HLA alleles between mother and child play a role (91), especially if the alleles are associated with rapid progression (92). Some children of HIV infected mothers that were not infected

produced higher amounts of chemokines that bind to CCR5 (93), potentially competing with HIV for binding to the co-receptor.

Disease progression in untreated HIV infected children is accelerated in comparison to infection of adults (94) with a median survival of only two years for vertically infected children compared to 11 years for PLHIV who acquired HIV later in life (95). It is speculated that this relates to the immaturity of the immune system at time of infection. However, the mode of transmission also influences progression, as children infected via blood transfusion experience slower disease progression compared to vertically infected children (96). This difference is in part caused by the similarities in HLA alleles, as the virus transmitted to the child is already adapted to the HLA alleles that are shared between the individuals (97).

Compared to HIV infection in adults, viral replication in pediatric HIV infection is much higher at peak level and declines slowly until the set point is reached at approximately 5 years of age (98). CD4 T cell levels are generally higher in children due to the development of the immune system, which might fuel the elevated replication as target cells are more abundant. However, T cell depletion in HIV infected children is progressive over the duration of infection instead of halting at a plateau as seen in chronic infection of adults (99).

Global efforts in diagnosing and treating pregnant women have reduced the number of vertical HIV infections and early initiation of ART for infected children has greatly improved survival. Screening of pregnant women has virtually eliminated MTCT in developed countries, essentially limiting vertical HIV infection to lower income countries, especially those with high incidence of HIV outside of risk groups. The problems regarding access to HIV testing and ART in those countries mean that diagnosis and treatment initiation of children is often delayed. In addition, regions where MTCT occurs are often struggling with other problems such as political conflicts, malnutrition, high infectious burden, but also stigma and discrimination of PLHIV. However, even in developed countries, pediatric HIV patients are at increased risk for virological treatment failure due to drug resistance (100), immunological failure or lower recovery of CD4 T cell count (101) and impaired vaccine responses (102).

Non-AIDS illnesses such as problems with vertical growth, muscle strength, neurological development, metabolic, and cardiac health affect physical development at a critical age and can thus lead to permanent disabilities (83). Comorbidities are mostly related to immune activation, which is in turn caused by simmering HIV replication below detection limits despite ART. Treatment thus decelerates comorbidities but does not restore health to levels seen in HIV negative individuals. Consequently, older age at ART initiation exacerbates both pathogenesis and comorbidities. Conflicting results on differences in immune recovery depending on treatment regimen led to guidelines that discourage treatment changes in case of suboptimal immunological outcome despite viral suppression. Furthermore, studies of new types of ART in children and adolescents is often delayed (103). The potential of early ART initiation is considered highly advantageous and even speculated to harbor the potential for cure (104), if optimal conditions are met. In search for curative therapies, factors such as

residual viral replication and the resulting immune activation, as well as the size of the viral reservoir are considered critical and should thus be investigated in pediatric infection. Studies have shown a reduced size of the viral reservoir in infected children on continuous ART (105), highlighting the potential of ART optimization in vertical HIV infection.

1.2.6 HIV-2 infection

HIV-2 infection differs substantially from the HIV-1 pandemic. On a nucleotide level, the viral genome of HIV-2 displays 55% sequence similarity with HIV-1, resulting in 55% similarity on protein level for Gag and Pol, and 35% for Env (106). HIV-1 and HIV-2 share transmission routes and cellular tropism, however cytopathogenicity and replication capacity might differ (107, 108) and transmission rates are lower in HIV-2 (109). Some differences between HIV-1 and HIV-2 infection are related to the viral genome, like sensitivity to tripartite motif containing protein 5 α (TRIM5α) (110), escape from inhibition of reverse transcription (111, 112), downregulation of the T cell receptor (TCR) complex by Nef (113), or responsiveness to human transcription factors (114).

In addition to host-virus interactions, the differences on genome and protein level have implications for diagnosis, monitoring and treatment (115). Diagnostic screening methods differ in their capability to detect HIV-2 due to variations in cross-reactivity between HIV-1 and HIV-2 depending on the technique and components used. Furthermore, quantification of HIV-2 VL is complicated by both the limited availability of detection kits and high frequency of natural control, suppressing plasma VL below detection limits. Similarly, ART is optimized for HIV-1 and HIV-2 infection is associated with problems like slow recovery of CD4 T cell counts and high prevalence of ART resistance, both before treatment initiation and in response to suboptimal ART. HIV-2 is inherently resistant to several classes of drugs (NNRTIs and the only fusion inhibitor available), and treatment with other drugs (several protease inhibitors) is less effective compared to HIV-1 infection. Furthermore, testing for additional resistance mutations is complicated in patients without detectable VL. Consequently, undetected HIV-1/HIV-2 dual infection can contribute to immunological failure on ART, but due to the low prevalence of HIV-2 this is not frequently the case in regions other than Western Africa.

Overall, HIV-2 infected individuals have lower viral set points and plasma VL compared to HIV-1 and the decline of CD4 T cells is slower (116-119). However, disease progression differs among HIV-2 infection, ranging from decade-long asymptomatic courses seen in a high proportion of viremic control (120, 121), to progression comparable to HIV-1 at similar VL and CD4 T cell counts (122, 123). Counterintuitively, AIDS onset in HIV-2 occurs at higher CD4 T cell levels when compared to HIV-1 (124, 125).

Similar to progression, immune responses and activation differ among HIV-2 infected individuals. Lower pathogenicity of HIV-2 compared to HIV-1 prevails at high to moderate CD4 T cell counts and low VL, but diminishes when VL increases and CD4 T cell counts are increasingly affected (126). Natural killer (NK) cell cytotoxicity (127), microbial

translocation (128), T cell activation (129), and sensitivity to apoptosis (130) differ between aviremic and viremic HIV-2 infection and further highlight the importance to distinguish between these groups. Lower CD4 T cell activation in aviremic HIV-2 compared to viremic HIV-2, HIV-1 and HIV-1/HIV-2 dual infection also extends to differences in the quality of CD4 T cells. Programmed cell death 1 (PD-1) expression, a sign of exhaustion of T cells, remains lower in aviremic compared to viremic HIV-2 (131).

In HIV-1 controllers, strong activation of both CD4 and CD8 T cells can suppress viral replication, but simultaneously contributes to depletion of CD4 T cells (132). The immune activation thus persists independent of viremia, and drives disease progression regardless. Similarly, aviremic HIV-2 infected individuals are not unscathed and still show pathogenic changes when compared to seronegative individuals and can progress to AIDS despite viral suppression (131). Interestingly, pre-existing HIV-2 infection even has the capacity to slow down HIV-1 disease progression in dual HIV-1/HIV-2 infection (133), which suggests that HIV-specific immune responses developed against HIV-2 can ameliorate HIV-1 already in acute infection before immune responses against HIV-1 can be mounted.

1.3 HIV IMMUNOPATHOGENESIS

The human immune system comprises two elements: components of the innate immune system recognize patterns that are common to pathogens, but not the human body, while cells of the adaptive immune system develop receptors that are unique to the specific antigen they are presented with. The development of the adaptive immune response requires genetic rearrangement based on coordinated antigen presentation and activating signals, a process that takes several days to reach potent efficacy. In contrast, the innate immune system relies on invariant receptors and cellular processes that can act immediately upon encounter of pathogens.

The main purpose of the immune system is to ensure human health by eliminating pathogens, keeping the symbiotic microbiome in check and avoid damage to host tissue in the process. Thus, a delicate balance between effective defense and prevention of tissue damage are essential. HIV infection debilitates both, impaired defense against infection and tissue damage due to increased inflammation are part of the immunopathogenesis.

1.3.1 Innate immune responses

Epithelial surfaces, such as skin and mucosa, are often considered part of the innate immune system, as they represent a physical barrier that protects the host from the majority of invading pathogens. The most common first encounter with HIV happens at mucosal surfaces, genital and rectal mucosa. DCs patrol skin and mucosa with the purpose of taking up pathogens, process them and present them to cells of the adaptive immune system. HIV is bound by dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) on the surface of DCs and taken up into vacuoles, where it can survive several days (21). In their pursuit to present the captured pathogen to immune cells, DCs migrate to draining lymphoid tissues, effectively presenting HIV with a high abundance of target cells.

DCs and other CD4 positive myeloid cell types are themselves rarely productively infected by HIV-1 due to SAM and HD domain containing deoxynucleoside triphosphate triphosphohydrolase 1 (SAMHD1) (111, 112). SAMHD1 inhibits HIV RT by restricting the access to deoxynucleoside triphosphates (dNTPs) required for DNA synthesis (134), thus preventing productive HIV infection. In contrast to HIV-1, HIV-2 possesses an accessory protein, viral protein x (Vpx), that interferes with SAMHD-1 mediated inhibition of HIV RT.

SAMHD1, apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3 (APOBEC3), TRIM5α, and tetherin are considered HIV-1 restriction factors. APOBEC3 is a cytidine deaminase, converting cytidine in viral RNA and DNA to uridine. This manipulation with the genome renders the virus incompetent. TRIM5α blocks HIV replication shortly after entering the cell by binding to the capsid protein. After budding of new HIV virions, tetherin retains the viral particle at the cell surface, preventing release of HIV virions from the infected cell. While these restriction factors restrain HIV infection, the virus counteracts restriction factors with the accessory proteins Vif, Nef, Vpu, and Vpr (135), enabling infection of myeloid cells (136).

In cells exposed to viral RNA and DNA, the viral components are bound by receptors of the innate immune system, primarily toll-like receptors (TLRs) and retinoic acid-inducible gene-I (RIG-I) like receptors. This interaction between pathogen associated molecular patterns (PAMPs) with pathogen recognition receptors (PRRs) is essential to sensing of viral infections. The ensuing signaling cascades stimulate expression of cytokines and chemokines to attract, activate and differentiate surrounding cells, thus initiating the immune response to HIV. As part of this response, interferon is released from the infected cell and induces expression of interferon stimulated genes (ISGs), including PRRs themselves, thus intensifying local inflammation (137). In a similar fashion, HIV-induced inflammation is exacerbated by co-infections and microbial translocation. Sensing of PAMPs from bacterial and viral sources adds to the signals originating from HIV infection, increasing inflammation and risk for comorbidities. As a result of microbial translocation, LPS in circulation is captured by CD14 on the surface of monocytes and macrophages, which are in turn activated via TLR 4. Soluble CD14 (sCD14) is thus a marker of monocyte activation (138) and an indirect marker of microbial translocation (139). Monocytes and macrophages respond by production of inflammatory cytokines and coagulation factors (140-142).

Successful recognition of PAMPs can abrogate HIV infection, but triggers pyroptosis in the course (143). The released cytokines attract and activate immune cells, and cytoplasmic contents serve as danger associated molecular patterns (DAMPs), which are additional triggers of innate immune responses and inflammation. Thus, pyroptosis is not only a major contributor to the depletion of CD4 T cells, but also the increased immune activation and inflammation seen in HIV pathogenesis (51).

The main PAMPs of HIV are the DNA and DNA:RNA products synthesized by HIV RT. Thus, RT inhibitors included in ART regimen can reduce inflammation from innate immune

responses (137, 144). Given the persistence of HIV, ensuring RT inhibitors to permeate into anatomical sites of residual replication could improve immune recovery and comorbidity burden in ART treated PLHIV.

NK cells are cytotoxic cells of the innate immune system that recognize tumor and infected cells. Their activation is mediated by interleukins, activated monocytes and macrophages, and their function is controlled by the net outcome of activating and inhibiting surface receptors. Killer immunoglobulin-like receptors (KIRs) on NK cells interact with HLA-C molecules, which drives their development and proliferation. High surface expression of HLA-C in PLHIV is associated with slower disease progression (145) and HIV develops mutations to escape from NK mediated immune responses (146), providing evidence for efficacy of immune response by NK cells. As cytotoxic cells, NK cells primarily combat HIV by inducing cell death of infected cells, but also shape the immune response by production of cytokines and chemokines (147).

Tissue-resident macrophages, which are differentiated from circulating monocytes, play an important role in tissue homeostasis. Their main function is phagocytosis of pathogens, apoptotic or stressed cells. Depending on the context, they can act either pro- or anti-inflammatory. In HIV, they are the second group of target cells in addition to CD4 T cells and might be part of the viral reservoir (148). When phagocytosing infected cells, they depend on signals like interferon-γ (IFNγ) and ligands on the CD4 T cell surface (21). When this CD4 T cell help is compromised, they can ingest, but no longer kill intracellular pathogens, which leads to the high prevalence of mycobacterial infections seen in AIDS.

In addition to their roles in containing infection directly, cells of the innate immune system are important in modulation and activation of the adaptive immune system. They process antigen and present it to B and T cells together with co-stimulatory signals and thus initiate development of antigen-specific receptors. Stimulation of the adaptive immune response improves after activation and maturation/licensing of innate antigen presenting cells (APCs) by optimizing the expression of co-stimulatory signals. In HIV, the persistence of inflammatory signals past the acute phase of infection promotes inhibitory mechanisms in an attempt to avoid excessive tissue damage. One prominent example is PD-1 (CD279) with the ligands PD-1 ligand 1 (PD-L1, CD274) and PD-L2 (CD273). In HIV infection, upregulation of PD-L1 was detected on cells of the innate immune system and even on the surface of HIV virions (149). Expression of the receptor PD-1 is increased in HIV infection both on NK cells and T cells (150, 151), rendering these cells receptive to inhibition by PD-L1 expressing cells and impairing the crosstalk between innate and adaptive immune system.

1.3.2 Cytokines and chemokines

The crosstalk between cells of the immune system is mediated by soluble and membrane bound proteins. Receptor-ligand interactions coordinate recruitment, activation and regulation of cells, and can determine which components of the immune system contribute to the response at hand. While acute viral infections lead to controlled cytokine and chemokine

(chemotactic cytokine) responses, limited both in their anatomical and temporal distribution, HIV causes a systemic and permanent increase in inflammatory signals (152, 153). This sustained dysregulated response contributes to the immunopathogenesis of HIV infection.

In an attempt to identify mechanisms of HIV protection or susceptibility, genome-wide association studies (GWAS) have advanced our knowledge in several aspects. The strongest protection found in these studies was a mutation of CCR5, CCR5Δ32, that results in a protein that R5-tropic HIV cannot bind to. People homozygous for this mutation are protect from HIV as it prevents viral entry and thus infection of target cells. PLHIV with heterozygous CCR5Δ32 experience partial protection as it is associated with slower disease progression.

CCR5 is a chemokine receptor with three ligands, C-C motif chemokine ligand 3 (CCL3), CCL4 and CCL5, also known as macrophage inflammatory protein-1α (MIP-1α), MIP-1β and regulated upon activation, normal T cell expressed and secreted (RANTES). By binding to CCR5 these chemokines can suppress HIV *in vitro* (154). Furthermore, they are elevated in people who are HIV negative despite exposure (155, 156), and both MIP-1α and MIP-1β have been associated with suppression of viral load (157). MIP-1β takes part in polyfunctional CD8 T cell responses against HIV (158). However, their roles in competing with HIV for entry on one hand and as inflammatory mediators on the other hand do create conflicting results, especially with the introduction of treatment. As chemoattractants, C-C chemokines increase inflammation by activating endothelial cells and recruiting immune cells such as lymphocytes, monocytes and DCs. MIP-1α is produced by a number of cells, especially stimulated immune cells. Innate immune cells such as monocytes and neutrophils produce both MIP-1α and MIP-1β upon encounter of LPS, other PAMPs and cytokines. Furthermore, HIV infected macrophages and CD4 T cells can secrete MIP-1α (159). In ART treated HIV, MIP-1β was linked to problems with immune recovery (160) and MIP-1α correlates with problems of lipid metabolism (161).

Interleukin-2 (IL-2) is a cytokine impacting a large number of hematopoietic cells. It induces proliferation of T cells, cell growth and survival. The potency of IL-2 mediated effects on CD4 T cells was investigated in clinical trials administering IL-2 to successfully treated PLHIV, which improved CD4 T cell counts. However, the resulting CD4 T cell activation also increased viral load (162) and IL-2 did not ameliorate clinical symptoms, possibly due to skewing features of CD4 T cells (163, 164).

Tumor necrosis factor α (TNFα), mainly secreted from activated macrophages and T cells, activates several innate immune mechanisms and is associated with numerous inflammatory conditions. Depending on the cell type receiving TNFα signals, it can downregulate CD4 and CCR5 and simultaneously upregulate MIP-1α, MIP-1β and RANTES as well as HIV restriction factors. However, intracellular signaling activates transcription and thus viral mRNA synthesis in infected cells. Furthermore, it has been implicated in apoptosis of CD8 T cells. TNFα signaling has thus a multitude of outcomes, that strongly depend on the cell types involved (165).

Another member of the TNF superfamily, TNF-related apoptosis-inducing ligand (TRAIL), is expressed by NK cells, T cells and DCs. In the context of HIV infection, it was associated with contribution to CD4 T cell death (166). TRAIL and other ligands of the TNF superfamily bind to so-called "death receptors" and induce apoptosis via effector caspases (167), or necroptosis in case of infections that inhibit apoptotic pathways (168). Necroptosis is another type of inflammatory cell death very similar to pyroptosis, but rather than a direct effect of PAMPs and stress signals, it results from abrogated apoptosis (169). The increase of TRAIL in HIV is ameliorated upon successful ART and *in vitro* experiments showed that cells from PLHIV are more sensitive to TRAIL mediated apoptosis than those from uninfected people (170).

IFNγ is produced in high levels by activated T and NK cells. It directly activates innate immune cells and influences CD4 T cell differentiation and indirectly enhances T cell responses by elevating peptide presentation. IFNγ levels in plasma of PLHIV is elevated and the cytokine contributes to HIV mediated inflammation. There are contrasting results regarding the influence of IFNγ on HIV disease progression, but it is often used as a marker for responses when evaluating HIV-specific immune responses in PLHIV or vaccine efficacy (171).

The majority of pro-inflammatory cytokines and chemokines are beneficial in acute HIV infection, but the sustained and dysregulated nature of immune responses in chronic infection causes HIV immunopathogenesis. The increased survival of PLHIV on ART uncovers resulting problems, such as immunosenescence and comorbidities.

1.3.3 Adaptive immune responses

The adaptive immune system encompasses B cells, CD8 T cells, the main target cells of HIV, CD4 T cells, as well as rare T cell populations such as NKT cells or γδT cells. CD8 T cells are the focus of this thesis and will be discussed in more detail in the following chapter (see 1.4 CD8 T cell responses).

B cells are responsible for antibody responses and depend on CD4 T cell help within lymphoid follicles in order to develop an appropriate antibody isotype and optimize affinity for their cognate antigen. B cells themselves are not infected with HIV, but bystander effects decrease serological memory in HIV (172, 173). However, B cells can bind virions through receptors like CD21, DC-SIGN and C-type lectin receptors. In addition, indirect effects via infected macrophages and most strikingly the lack of CD4 T cell help affect B cells. HIV mediated immunopathogenesis leads to both activation and exhaustion of B cells, resulting in reduction of resting B cells and increases in activated and transitional memory subsets. Transitional B cells are weakly responsive to antigen stimulation and more sensitive to apoptotic signals like CD95. Exhaustion of B cells results in lower epitope diversity and proliferative capacity as well as senescence. HIV mediated B cell pathogenesis thus results in increased turnover and malignancies of B cells, and hypergammaglobulinemia with simultaneous loss of serological memory to vaccines (173).

CD4 T cells**,** also called helper T cells, are central to coordinating and shaping the appropriate immune response by providing support to other immune cell types. They recognize peptides that are processed and presented by HLA class II molecules on professional APCs. These cells are themselves immune cells and receive help in the form of membrane bound ligands and secreted cytokines by activated CD4 T cells. This crosstalk ensures optimal effector function, like phagocytosis by macrophages and maturation of DCs, as well as differentiation and optimization of adaptive responses, like class-switch of B cells and maintenance of CD8 T cell memory. CD4 T cells differentiate into several subsets that balance the contribution of immune system compartments to the overall response by selectively boosting one cell type and suppressing others. Th17 cells are integral to homeostasis of the gut barrier function and regulatory T cells counteract tissue damage and maintain immune tolerance to signals like self-antigen or the symbiotic microbiome. In addition to depletion of regulatory CD4 T cells (174), which diminishes suppression of inflammation, the dysregulated cytokine environment also interferes with development of regulatory T cells (175). Both these subsets are strongly affected by HIV mediated CD4 T cell depletion and their loss contributes to HIV immunopathogenesis.

HIV requires the cellular transcription machinery for viral mRNA synthesis. Transcription factors like nuclear factor κB (NFκB) and nuclear factor of activated T cells (NFAT) are only upregulated upon activation, and their absence in resting CD4 T cells results in latent infection. However, the highly inflammatory environment created by HIV causes excessive CD4 T cell activation and thus viral replication and cytopathic depletion.

B cells receive CD4 T cell help by T follicular helper cells (T_{FH}) in lymph nodes and this interaction depends on fibroblast reticular cells. In their attempt to curb the ongoing inflammation, regulatory T cells release transforming growth factor β (TGFβ), which results in fibrosis of lymph nodes. Fibrosis, the deposition of collagen, however replaces the fibroblast reticular cells and thus disrupts interactions between B cells and T_{FH} (153). The subsequently dysregulated B cells are an additional source for TGFβ and create a vicious cycle culminating in destruction of the lymph node architecture (176). Furthermore, T_{FH} were linked to persistent viral replication despite treatment (66) and are a proposed component of the viral reservoir.

CD4 T cells also mount HIV-specific responses. Furthermore, elevated C-C chemokine levels produced by the HIV-specific CD4 T cell response were detected in individuals that were exposed to HIV, but remained uninfected (155) as well as long-term non-progressors (177).

HIV infection stimulates proliferation of memory T cells and decreases the naïve pool of both CD4 and CD8 T cells. However, among CD4 T cells depletion dominates, while the CD8 T cell pool expands (178). The result is a skewed ratio of CD4 to CD8 T cells that reliably predicts disease progression and pathogenesis (179-181).

1.4 CD8 T CELL RESPONSES

1.4.1 Successful T cell responses

The majority of CD8 T cells in humans express αβ TCRs and are part of cytotoxic antigen-specific responses. With their TCR, CD8 T cells recognize antigen presented in the form of a peptide/major histocompatibility complex (MHC) I molecule complex on the target cells. The epitope is a peptide, typically 9 – 11 amino acid long, bound to the peptide binding groove of HLA class I molecule, the human MHC I protein. This interaction between TCR and the peptide/HLA complex is the prerequisite for T cell activation, but it is not sufficient for full activation of CD8 T cells. Upon their first encounter with antigen, naïve CD8 T cells are typically presented with antigen in the context of other activating signals by DCs, which results in priming of the CD8 T cell. Naïve CD8 T cells express ligands such as CD28, which binds to CD80 or CD86 on APCs. This combination of TCR and co-stimulatory signals leads to priming or, in the case of differentiated T cells, execution of their respective functions. In the case of naïve T cells, priming results in strong proliferation and thus generation of both effector and memory T cells.

Effector CD8 T cells are guided by chemokines to migrate to the infected or malignant tissue and carry out the primary response. Antigen recognition at the site of infection does not require the same extent of co-stimulation that induced priming (21, 182). Upon binding to a cell presenting the cognate antigen, effector CD8 T cells form an immunological synapse with the infected target cell. The resulting polarization transports cytotoxic granules towards this contact, where their contents are released in a targeted fashion. Perforin penetrates the cell membrane of the infected cell, enabling granzymes to enter the cell and trigger apoptosis (21). In addition, antigen recognition initiates *de novo* expression and secretion of cytokines such as IL-2, TNFα and IFNγ. The simultaneous expression of various effector molecules is termed polyfunctionality and an important aspect of successful CD8 T cell responses. Effector CD8 T cells are short-lived, resulting in contraction of the immune response after an infection is resolved.

Memory T cells are a small subset of the primary response that provides long-term protection from previously encountered pathogens (183), but their survival is dependent on CD4 T cell help (184). Distinct subsets of memory T cells are associated with different functions, homing and thus expression of surface markers that are used for identification. In blood, memory T cells are usually characterized as central, effector or terminal effector memory T cells. Central memory T cells react to antigen exposure by proliferation, thus giving rise to immune responses upon second encounter with a pathogen. They still express the co-stimulator CD28 and C-C chemokine receptor 7 (CCR7), which is associated with homing to lymph nodes. In contrast, effector memory T cells migrate to inflamed tissues and can rapidly execute effector functions. They have downregulated both CD28 and CCR7. Another marker used to distinguish T cell subsets is the phosphatase CD45. Naïve T cells express the isoform CD45RA, which then changes to CD45RO expression in central and effector memory T cells. However, terminal effector memory T cells express CD45RA and are also called TEMRA

cells, after their re-expression of CD45RA. With the use of more markers, additional subsets can be distinguished. For example, transitional memory T cells display an intermediate differentiation state between central and memory T cells. Transitional memory T cells have downregulated CCR7 but are still positive for CD28. Apart from circulating memory cells, tissue-resident memory T cells provide local immune responses and can be identified by the expression of tissue homing receptors.

Surface protein expression changes in response to antigen encounter, which is often used to identify and quantify recently activated T cells. Some co-stimulators, such as CD226, OX40 (CD134) or 4-1BB (CD137), are only upregulated as response to activation. However, changes in protein expression mediate a number of adaptations that distinguish resting from stimulated cells. CD38 is an enzyme with roles in cell adhesion, signal transduction and calcium signaling, and HLA-DR is an MHC class II molecule that mediates interaction with CD4 T cells. Antigen encounter results in proliferation of T cell subsets why proteins with roles in the cell cycle, such as Ki67 (185), but not expressed in resting cells, are typically used to quantify proliferation in response to T cell activation. CD69 is rapidly expressed upon stimulation but downregulated shortly after. Thus, CD69 is often used as an activation marker, but the physiological role is less clear. It has been suggested, that CD69 might restrain inflammation (186).

In order to avoid tissue damage, autoimmune disorders as well as vascular damage, and resolve immune responses after acute infections, T cells also express co-inhibitory receptors (also called immune checkpoints) (187). Many of these inhibitory receptors share the same ligand with co-stimulators, resulting in direct competition. In an appropriate T cell response, co-inhibitors are thus upregulated as a direct result of T cell activation and often serve as activation markers in T cell biology. As pathogen is cleared and antigen exposure ends, effector T cells die by neglect, as they do not receive survival signals any longer. The remaining memory T cells decrease the expression of proteins associated with activation, including co-inhibitory receptors.

The first activation of CD8 T cells is seen even before peak viremia and both rapid and abundant CD8 T cell responses are associated with a lower viral set point in chronic infection (188). This has implications for the clinical outcome, as peak viremia and the viral set point are linked to disease progression. Furthermore, the need for HIV to escape CD8 T cell responses visible by mutations in viral epitopes documents efficacy of the HIV-specific response (189, 190). However, the initial response of HIV-specific CD8 T cells does not succeed in clearing all virus-infected cells and thus viral replication remains after acute infection.

Despite the connection of polyfunctionality and cytotoxicity of circulating CD8 T cells to better disease outcome in HIV, new studies have revealed that this is not the case in tissue. Non-cytolytic CD8 T cell responses in lymph nodes and other tissues (191) are beneficial for HIV-specific immune responses and highlight the importance of anatomical location and adaptation of the cells to their surroundings (192).

1.4.2 T cell exhaustion

Persistence of antigen stimulation in chronic infections and cancer lead to T cell exhaustion. First studied in a murine model, the loss of IFNγ and reduced magnitude of a virus-specific response in chronic infection were noted (193). In addition to viral persistence, loss of CD4 T cell help exacerbates T cell exhaustion (194). The continuous antigen exposure maintains and increases expression of inhibitory receptors and drives progressive loss of proliferative capacity and polyfunctionality, as well as changes in the transcription factor profile, epigenetic landscape and metabolism of the affected T cells (195-198). Even though antigen exposure is the main driver of T cell exhaustion (199), viral suppression with ART can only slow down, but not completely halt upregulation of inhibitory receptors, which is probably related to the residual replication.

PD-1 was among the first inhibitory receptors described in exhaustion and more specifically in HIV (200-202). Subsequent studies implicated several other inhibitory receptors, such as cytotoxic T-lymphocyte associated protein-4 (CTLA-4), killer cell lectin like receptor G1 (KLRG1), CD160 and CD244 (2B4) both in cancer and HIV (203). The upregulation of inhibitory receptors can progress in two aspects, increasing the expression level of any given inhibitory receptor, and the accumulation of multiple receptors per cell. The expression level can determine whether exhaustion is reversible, as is the case for cells with low, but not high levels of PD-1 (204, 205) and receptors can synergize in their inhibitory function, as demonstrated for T cell immunoreceptor with Ig and ITIM domains (TIGIT) and PD-1 (Fig. 2) (206, 207). Apart from their expression on T cells, the impact of inhibitory receptors is also determined by the expression of their ligands on surrounding cells, as mentioned for the ligands of PD-1 on innate immune cells (1.3.1. Innate immune responses).

In addition to the synergy with PD-1, TIGIT counteracts co-stimulation mediated by CD226 (208), which is required for T cell activation by non-professional APCs (209). TIGIT and CD226 share a ligand, poliovirus receptor (PVR, CD155), for which TIGIT has a higher affinity than CD226 (Fig. 2). Furthermore, TIGIT disrupts dimerization of CD226 (206), which is necessary for co-stimulatory signaling. In chronically HIV infected individuals, TIGIT expression is increased (210) and expression of CD226 is decreased (211), suggesting that the TIGIT/CD226/PVR network contributes to exhaustion mediated by HIV.

In contrast to TIGIT and PD-1, both of which possess immunoreceptor tyrosine-based inhibitory motifs (ITIMs), 2B4 possesses an immunoreceptor tyrosine-based switch motif (ITSM), which enables the receptor to take on either a co-stimulatory or an inhibitory role (212). Most insight into the functions of 2B4 stem from NK research, where it was shown that the expression level of 2B4 and the availability of both the ligand CD48 and adaptor molecules determine about the signaling outcome of 2B4 (212). In addition, 2B4 was shown to synergize with CD226 in a co-stimulatory capacity (213).

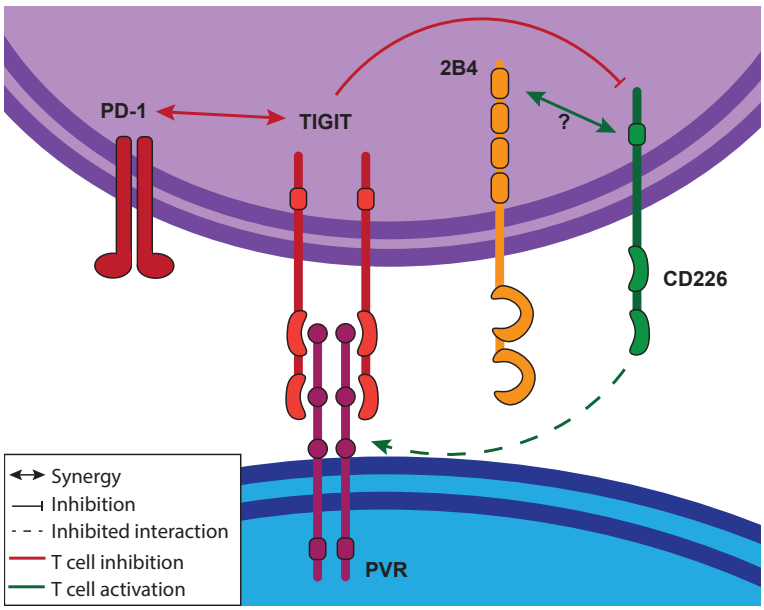

Figure 2. Interactions between regulatory networks analyzed in this thesis.

Another hallmark of T cell exhaustion is the gradual loss of polyfunctionality of antigen-specific T cells. IL-2 is a potent cytokine in T cell responses against HIV (214, 215) and among the first effector molecules lost in exhaustion (195). IL2 promotes T cell homeostasis by induction of proliferation, which directly links it to the CD4 T cell count in PLHIV. In contrast, IFNγ expression and degranulation are features that are maintained until late stages of exhaustion. Especially degranulation of empty granules when expression of cytotoxic molecules has already been lost is associated with ineffective T cell responses in HIV (216).

The transcription factors T-box transcription factor 21 (T-bet) and eomesodermin (Eomes) regulate effector function and memory survival of CD8 T cells (217). They seem somewhat mutually exclusive, as high expression of either is associated with intermediate levels of the other, giving rise to T-bet^{hi}/Eomes^{dim} and T-bet^{dim}/Eomes^{hi} transcription profiles. T cell exhaustion in HIV has been linked to a shift towards the T-bet^{dim}/Eomes^{hi} profile (218). However, T cell exhaustion seems to be initiated early in the differentiation process and recent insights into the transcription factor and epigenetic landscape of T cells has linked a progenitor subset of exhausted T cells to the transcription factor thymocyte selection associated high mobility group box (TOX) (219).

While many mechanisms and key players of T cell exhaustion are shared between CD4 and CD8 T cells, some differences remain. Based on development and differentiation, the functional markers for CD4 and CD8 T cells are inherently different. Furthermore, the specific inhibitory receptors that accumulate on the cell surface depend on the cell type, as well as environmental factors. CTLA-4, hepatitis A virus cellular receptor 2 (HAVCR2, better known as Tim-3) and lymphocyte activating 3 (Lag-3) are readily detectable on CD4 T cells, but much less abundant in HIV-mediated CD8 T cell exhaustion.

1.4.3 Influence of HLA-alleles on T cells and HIV infection

Some of the strongest and most consistent genes associated with protection in HIV GWAS studies are HLA class I genes, especially HLA-B*57:01 (220, 221). Their role in antigen presentation to CD8 T cells directly implicates CD8 T cell responses in this protection. In contrast, some other HLA genes are linked to susceptibility to and rapid progression of HIV infection, demonstrating the devastating effect of impaired HIV-specific CD8 T cell responses (222, 223).

Proposed mechanisms for the influence of HLA genes include structural features of the peptide binding groove, which determines the peptide sequences that can be presented and thus the capability to present conserved sequences (*e.g.* from HIV-Gag p24). Mutations in the targeted conserved HIV region to escape the associated immune response dramatically diminish virulence or fitness of the virus and thus slow down disease progression (224). Certain HLA alleles might also promote the development of cross-reactive or high affinity TCRs (225), thus shaping the T cell repertoire. Furthermore, decreased sensitivity to regulatory T cell suppression by HLA-B*57 was demonstrated (226), causing stronger immune responses in general. This might be one factor in the association of HLA-B*57 positive PLHIV with slower disease progression, autoimmune comorbidities and lower prevalence of hepatitis C virus (HCV) (227).

In the context of HLA-B*57 in HIV infection, protection has also been linked to the specific epitopes targeted (228, 229), high-magnitude HIV-specific responses early in infection (230), cross-reactivity (231), as well as the capability to target viral mutations with CD8 T cell responses that include IL-2 and perforin (232).

However, the extent of protection conferred by HLA-B*57 is not equal among these individuals and comparing PLHIV with different progression rates (214, 233) implicate a complex interplay of virus and host interaction in this difference that is not fully understood yet.

1.5 CURRENT CHALLENGES IN HIV RESEARCH

1.5.1 Comorbidities

For PLHIV accessing ART, especially when initiated timely, life expectancy is now close to that of the HIV negative population (234). However, comorbidities, or non-AIDS-defining illnesses, reduce quality of life despite viral suppression and are increasingly the cause of death among PLHIV (235, 236). Comorbidities include chronic lung disease, frailty syndrome, bone disease, neurological symptoms, immunologic ageing, renal impairment, dermatologic manifestations, cardiovascular and liver diseases as well as malignancies (83, 237, 238).

The main drivers for these complications are adverse effects of ART, co-infections and residual HIV replication causing immune activation and deterioration. However, factors like disease severity at ART initiation, older age, as well as life-style related risk factors such as

alcohol consumption and smoking, influence the development of comorbidities. Some of these risk factors and mechanisms are universal, while others differ between populations. Other infectious diseases and delayed ART initiation are more prevalent in low and lower-middle income countries, while air pollution is often associated with urban settings. Malnutrition and CMV infection are important factors in pediatric HIV infection and exacerbate comorbidities that interfere with development (83).

In the general public, the prevalence of metabolic syndrome increases due to modern life-style changes (239). However, HIV infection and ART, especially early drugs, increase the risk of insulin resistance and abnormal lipid metabolism, even in children (240-243). Metabolic syndrome affects numerous organs, especially liver and kidneys, as well as fat distribution. In combination with immune activation, metabolic syndrome also causes endothelial dysfunction, atherosclerosis and hypertension, which in turn increase the risk for myocardial infarction and stroke (244), as well as neurological disorders like dementia (245).

Overall, HIV-mediated immune dysregulation leads to increased pro-inflammatory signals and decrease of immunomodulatory, or anti-inflammatory processes. Cytokines, like IL-6 and interferon-inducible protein 10 (IP-10, CXCL10), soluble biomarkers that result from monocyte and macrophage activation (246), components of the complement system and coagulation factors are elevated in plasma of PLHIV (247). Furthermore, oxidative stress and dysregulation of reactive oxygen species contribute to inflammation and tissue damage (248). Co-infections, especially other chronic viral infections such as CMV, HBV, HCV and EBV (249-251) also contribute to the chronic inflammation. As a result of immune activation and dysregulation, the immune system, especially the T cell compartment, of PLHIV shows signs of immunosenescence normally seen in advanced age (142), such as upregulation of the senescence marker CD57 (252) and shortened telomere length (253).

Chronic inflammation results in tissue damage of numerous organs and interferes with their physiological functions. Reduced thymic function, a contributor to the loss of CD4 T cells, does not recover with initiation of ART (254). Disruption of the gut epithelium, resulting in microbial translocation (255), also strongly influence comorbidities. Common complications in PLHIV associated to microbial translocation and thus elevated sCD14 concentrations are cardiovascular disease (256) and inadequate CD4 T cell restoration on ART (257).

Given the numerous mechanisms in which chronic inflammation contributes to development of comorbidities, evidence for cumulative effects of low grade replication despite ART on comorbidities, immune recovery, and disease progression (258, 259) is not surprising and stresses the importance of viral suppression. Vertically infected children are facing a life with the chronic infection, associated lifelong treatment and pathogenesis and cumulative exposure to HIV and ART immune activation (260). The higher viral load in vertically infected children increases T cell activation (261) and thus exhaustion (202) compared to adults. Together with disturbances of the B cell compartment, this decreases response to vaccinations (262, 263). Given the evidence that disease continues to progress despite treatment and

suppressed virus levels, these children are at much higher risk of several non-AIDS events, including atherosclerosis and metabolic syndrome, at a young age (264).

HIV is not directly carcinogenic, but the immunosenescence/immunosuppression, co-infection with oncogenic viruses and increased inflammation further contribute to the elevated risk of cancer in PLHIV (265, 266). Depending on their association with disease severity, malignancies are classified into AIDS-defining cancers and non-AIDS-defining cancers (251). While the risk for AIDS-defining cancers decreases with ART, non-AIDS defining cancers are more common due to increased survival of PLHIV.

1.5.2 HIV cure research

To end the HIV pandemic, research focusses on two approaches that complement one another: prevention of new infections by vaccination or other preventative methods as well as curative therapy. Currently, the increase in drug resistant HIV, requirement for life-long adherence and limitations in access to both diagnosis and treatment are major challenges in the fight against HIV and would greatly benefit from both vaccines and curative therapy.

HIV vaccines, if effective, would hold an enormous promise in fighting the spread of HIV. Although antibodies against HIV Env develop in the vast majority of patients, the high mutation rate of HIV leads to rapid escape and viral evolution of the targeted protein. Most of the conserved parts of Env, and thus promising epitopes, are shielded by glycan residues and consequently inaccessible for antibodies. However, broadly neutralizing antibodies, targeting epitopes that are conserved not only across escape mutations of autologous viral variants but also across HIV-1 clades, have been isolated from patients. These antibodies typically require two to three years of infection to develop, as they are the result of extensive somatic hypermutation, show unusual characteristics (267), and target one of only six epitopes of HIV Env (268). However, both the potency and breadth of these antibodies vary and could be used in different ways. The most potent antibodies neutralizing the broadest range of HIV variants might not be a realistic target for vaccine strategies, but passive transfer for both prophylaxis and therapy showed encouraging results in animal models and early human trials (268). However, HIV rapidly develops resistance to administration of a single antibody, demonstrating the need for combination therapy as used with current ART drugs.

In contrast to adult patients, vertically infected infants can develop broadly neutralizing antibodies as early as one year after infection (269). Development of these antibodies was associated to infection with at least two variants of HIV, highlighting the importance of exposure to diverse viral sequences. Reduced diversity of viral sequences in addition to the time needed for extensive somatic hypermutation might contribute to the long time required to develop broadly neutralizing antibodies in adults. However, infection with two HIV strains is still not sufficient to broaden antibody responses in adults the same way as in children (269), suggesting contribution by unique features of the developing immune system to antibody development.

Curative treatments have two potential outcomes: functional and sterilizing cure (270). The latter is defined as the complete elimination of pathogen from the host and might not be achievable for HIV due to the viral reservoir. A functional cure, or HIV remission, would entail long-term control of both viral replication and disease progression without the need for medication. Elite controllers might mimic functional cure, as they are control viral replication and maintain CD4 T cell count for years, but low grade replication and immune activation lead to comorbidities and prove that they are not unscathed either (271). So far, only two patients were cured from HIV infection. Both patients received stem cell transplants that replaced their immune system with hematopoietic cells harboring the homozygous CCR5Δ32 mutation (272, 273). To circumvent the need for rare compatible donors with the homozygous mutation, *in vitro* gene editing of CCR5 using zink finger nuclease or short hairpin RNA was used to either introduce double-strand breaks in the gene, mimicking the CCR5Δ32 mutation, or downregulate CCR5 on stem cells. However, so far none of these approaches has led to durable results (274). In one clinical study, gene editing of CCR5 with clustered regularly interspaced short palindromic repeats (CRISPR) resulted in successful engraftment, but low frequency of the edited gene (275). Other approaches aim at using CRISPR to directly target HIV sequences in infected cells (276).

As the longevity of the viral reservoir is the main challenge in preventing cure of HIV, several ways have been explored to either address or exploit it. The size of and mechanisms establishing the latent reservoir, as well as cell populations that contribute to either latent infection or viral rebound after treatment interruption are among the key questions in targeting viral persistence. Studies of ART intensification (addition of a fourth or even fifth drug to standard triple therapy) (277) or very early ART initiation (278) suggest that it is indeed possible to reduce, though not eliminate, the viral reservoir. Latency reversing agents (LRAs) are hypothesized to reactivate latent virus, allowing elimination by immune responses, ART or cytopathogenicity and thus “flush out” the reservoir (279). Thus far no single LRA has shown sufficient results at tolerable doses (280).

A similar approach is the use of immune checkpoint inhibitors (281). These inhibitors have been successfully used in cancer immunotherapy to reverse T cell exhaustion and induce immune responses clearing tumor cells. Monoclonal antibodies against inhibitory receptors or their ligands disrupt interaction of the binding partners without triggering the inhibitory signal, consequently reinvigorating T cell responses (282). However, only a subset of cancer patients responds to checkpoint blockade and this response is associated with severe immune-related adverse effects. Nevertheless, success in malignancies with poor prognosis has led to approval of monoclonal antibodies blocking interactions of PD-1 and CTLA-4 with their respective ligands, and further antibodies are in development (283). In regard to HIV, checkpoint blockade is hypothesized to aid a “shock and kill” concept (270, 280). Diminishing inhibition of CD4 T cells simultaneously facilitates T cell responses and reactivation of latently infected cells. Furthermore, direct effects on CD8 T cells expressing the same inhibitory receptors as well as indirect effects via CD4 T cell help should boost HIV-specific CD8 T cell responses and thus promote elimination of infected CD4 T cells.

Apart from T cells, NK cells also benefit from the improved CD4 T cell help (284). Ideally, checkpoint blockade would disrupt the pathogenic mechanisms of HIV infection and lead to a durable stalemate between virus and immune system, with minimal overall immune activation but highly effective responses targeted against HIV. A pioneering study used PD-L1 blockade in PLHIV recruited patients with residual, but still detectable viral load below standard detection limit (285). In two of the six patients, HIV-specific CD8 T cells responded with production of IFNγ, TNF or degranulation. Response in only a fraction of treated individuals is reminiscent of cancer therapy and might reflect expression levels of PD-1 (286). High expression levels of PD-1 render exhaustion irreversible, while lower expression levels allow for reinvigoration by checkpoint blockade.

Contrary to reactivation, the "block and lock" approach is based on the concept of "deep latency" (287). Latency promoting agents are supposed to durably suppress HIV and prevent future viral rebound currently seen upon ART cessation. However, inactivation of CD4 T cells resulting from this approach might exacerbate immunosuppression of PLHIV.

Another approach inspired by cancer immunotherapy is the generation of chimeric antigen receptor (CAR) T cells (288), which combine the antigen binding domain of antibodies with intracellular signaling domains of the TCR/CD3 complex. Further T cell based approaches aim at harnessing γδ T cells, non-cytolytic CD8 T cells or modulate T cell function with cytokine therapy (289).

2 RESEARCH AIMS

The overall aim of this thesis was to gain more insight into the immune deterioration seen in HIV infection, both on a systemic level caused by immune activation and inflammation, and on HIV-specific CD8 T cells as a direct consequence of the HIV-specific immune response.

Paper I: The inhibitory receptor TIGIT had recently been described in cancer immunotherapy research. Our aim was to investigate the role of TIGIT during T cell exhaustion in HIV infection and it´s relation to the transcription factors T-bet and Eomes in PLHIV cohorts with different disease and treatment status.

Paper II: Protective HLA alleles are associated with slower disease progression and effective HIV-specific CD8 T cell responses. We hypothesized that this association extends to less evident exhaustion of HIV-specific CD8 T cells restricted by the protective HLA allele in early untreated infection. To this end, we analyzed polyfunctionality, inhibitory receptors and transcription factors of responses restricted by HLA-B*57 or other HLA alleles.

Paper III: Viral replication in HIV-2 infection is often controlled by the immune system. However, progression to AIDS in HIV-2 infection can occur despite controlled viremia. We set out to describe activation and exhaustion of CD8 T cells in HIV-2 infection with or without detectable levels of viremia.

Paper IV: Perinatally infected children are a small proportion of PLHIV with higher risks for a number of comorbidities and complications than adult PLHIV. We set out to analyze plasma biomarkers of immune dysregulation in a cohort of HIV infected children in Sweden.

3 MATERIALS AND METHODS

3.1 PATIENT MATERIAL

To describe details of TIGIT expression on circulating CD8 T cells and the influence of ART for **paper I**, blood samples from PLHIV under care in Swedish Hospitals (Karolinska University Hospital Huddinge and Stockholm South General Hospital) were drawn. These represent untreated PLHIV (n = 30) and PLHIV on long-term ART (n = 20, treatment > 6 years, viral suppression > 5 years). Samples from acute infection (n = 12) and elite controllers (n = 14) were retrieved from the OPTIONS (290) and SCOPE (291) studies in the US (San Francisco General Hospital). Healthy control samples were recruited in Stockholm (n =26).

HIV diagnosis as early as acute infection is rare. However, regular screening of risk groups in countries where the majority of HIV infections are associated with these populations enable studies of acute infection. Elite controllers represent outliers with regards to disease progression and while the exact number depends on the criteria used, they make up less than 1% of PLHIV (292). As new infections with HIV decrease over the years, recruitment of such rare patient populations requires involvement of large cohort studies and programs.

To evaluate PVR expression within lymph node resident cells, paired lymph node and blood samples from PLHIV and HIV negative control subjects were obtained from the National Institute of Respiratory Diseases, Mexico City, and the University of Pennsylvania, US, respectively. In contrast to peripheral blood samples, which are associated with very low risk, collection of lymph nodes poses a larger risk for the individuals. Biopsies without a medical reason, as in well treated PLHIV and healthy people, are hard to justify ethically, but medical reasons for lymph node biopsies are typically connected to diseases that interfere with the studies at hand. One such example is the removal of adjacent lymph node during cancer surgery. While there is a medical reason (determining whether malignant cells have metastasized), cancer does have a major influence on the immune system with an emphasis on T cell exhaustion, much like HIV.

Paper II investigates the influence of the HLA-B*57 allele on CD8 T cell responses over the course of chronic infection. Peripheral blood mononuclear cells (PBMCs) from six HLA-B*57 positive and six HLA-B*57 negative PLHIV from the OPTIONS (290) cohort were obtained. Healthy control samples were recruited in Stockholm, Sweden.

The individuals involved in this study stayed mostly untreated (except for temporary treatment in two individuals). Since the introduction of triple combination ART (available in 1996), recommended treatment initiation has been revised regularly with rising CD4 T cell count thresholds. Since 2015 the world health organization (WHO) recommends treatment for all PLHIV regardless CD4 T cell count. In the course of research studies, treatment conforming with standards of the time has to be offered and recommended to the study participants. Thus, studies of longitudinal untreated PLHIV is not possible beyond 2015. However, based on the regulations at the time of sample drawing, the participants in the

OPTIONS cohort had the choice to either start treatment at a CD4 T cell count of 350 cells/mm^3 or choose to initiate ART right at diagnosis.

Comparison of groups of HIV-2 infected individuals in **paper III** was performed with blood samples drawn from an occupational cohort in Guinea-Bissau (293). In addition to the HIV negative control group (n = 27), PLHIV groups included HIV-1 infection, ART naïve or treated with virological failure (n = 12), successfully treated HIV-1 infection (n =8, VL < 75 RNA copies/ml), HIV-2 infection (n = 23) and HIV-1/HIV-2 dual infection (n = 5). For more detailed analysis, HIV-2 infected PLHIV were divided into viremic (n = 9) and aviremic (n = 14, VL < 135 RNA copies/ml).

Given the large proportion of viral control and asymptomatic disease course of HIV-2 infection, recruitment at medical health care facilities will underrepresent aviremic HIV-2 infection. In addition, geographic and genetic factors greatly impact the immune system and need to be considered when recruiting HIV negative control groups. The frequency of exposures and nature of pathogens encountered in different parts of the world as well as alleles, polymorphisms and differences in copy numbers of genes shape the human immune system considerably. Thus, choices regarding the recruitment of control subjects can impact study results. Individuals of this cohort are recruited via their place of work and screened for infections in retrospect, avoiding bias towards symptomatic individuals among PLHIV.

In **paper IV,** we evaluated immune activation in vertically HIV infected children (n = 20, 4 – 14 years of age) and adolescents (n = 14, 16 – 20 years of age), as well as control groups of HIV negative children (n = 17, 2 -15 years of age) and horizontally HIV infected adults (n = 51). Blood samples were collected at Karolinska University Hospital Huddinge.

Studies involving individuals underage require heightened emphasis on information and consent. The informed consent in studies involving children is given by parents or guardians and the child when possible. In addition, the vast majority of pediatric HIV infections in Sweden are children who immigrated to Sweden after birth. Language problems and being unfamiliar with local regulations can be the source of misunderstandings that need to be avoided. Furthermore, recruitment of the control group comprising HIV negative children faces problems like matching age, ethnicity and/or country of origin, as well as extra blood vials not collected for treatment or monitoring purposes. To mitigate the addition of harm or risk, volume of medically indicated blood drawing was increased by the sample used in our study. For this reason, HIV negative subjects included children either with medical conditions other than HIV infection, such as agammaglobulinemia or conditions affecting iron metabolism, followed up after recovering from a disease, or siblings of PLHIV. Exclusion of infectious diseases or conditions with known impact on the immune system is crucial, but the recruited group still represents individuals differing from the age-matched general public with regards to their health status.

3.2 FLOW CYTOMETRY

Multicolor flow cytometry is a single cell method widely used in cell biology, especially immunology. Cells are stained with fluorescent probes, most commonly antibodies coupled to fluorescent dyes, and analyzed for attached or internalized probes. The dyes are excited with the use of lasers and the emission of photons is detected after passing through filters to separate them by wavelength and photomultipliers to adjust the intensity of the signal to an appropriate range for quantification. Due to spectral overlap of the excitation and emission wavelengths, the number of parameters analyzed simultaneously is restricted by ways of distinguishing between the dyes. Separation of the signals is achieved by using multiple lasers and filters with defined wavelengths, minimizing overlap of excitation and emission spectra of the dyes in the experiment design and during subsequent analysis (compensation). Improvements of the involved components continue to increase the number of simultaneously detected parameters and thus dimensionality of the data. Analysis at single cell level is achieved by using cell suspensions at concentrations that allow cells to pass through a fluidics system one by one.

3.2.1 Experimental designs

Analysis of HIV-specific T cells in **paper I and II** was achieved by two different experimental designs. The main method used was stimulation with peptides, either a pool of peptides covering a defined region of viral genomes, or well described epitopes contained in the patient´s autologous viral sequences and presented by their HLA alleles. HIV-specific T cells were then identified by the means of antigen-specific responses like IFNγ expression, or extracellular localization of CD107a, a sign of degranulation. Furthermore, responses are defined as a minimum of 0.05% responsive cells after subtraction of the background signal in corresponding unstimulated samples, and at least twice the background signal. Alternatively, tetramers were used. These are protein complexes of four HLA molecules presenting defined epitopes and coupled to fluorescent dyes. Peptide stimulation limits the identification of T cell responses to cells expressing at least one of the functional markers, thus leaving out terminally exhausted cells, and might change expression of some markers by activating the cells. Use of peptide pools identifies a wider range of T cell responses by covering several epitopes included in the pool and processed and presented by the patient´s PBMCs. Epitopes represent a more narrow immune response and allowed to distinguish between responses restricted by either HLA-B*57 or other alleles. In contrast, use of tetramers is limited to the combinations of HLA alleles and viral sequences available either commercially or from collaborating laboratories, but has the benefit of identification of all specific T cells, including those incapable to express functional markers.

Blood samples for CD8 T cell analysis in **paper III** were collected in Cyto-Chex BCT tubes (Streck) due to the limited infrastructure at hand in Guinea-Bissau. The blood collection tubes do contain preservatives that allow for sample integrity despite storage at room temperature for 14 days before processing (294). However, the preservation interferes with cell functions, thus peptide stimulation and functional analysis was not possible with these samples.

Markers used in flow cytometry included extracellular markers like the lineage markers CD3, CD4 and CD8 for basic identification of CD4 and CD8 T cells, differentiation markers, inhibitory and co-stimulatory receptors, as well as intracellular proteins like effector molecules and transcription factors, depending on the scientific question addressed in the paper.

3.2.2 Data analysis and presentation

Integration of the single cell analysis from flow cytometry, clinical information and comparison of multiple conditions per sample/patient (*e.g.* peptide stimulations) require advanced bioinformatics to handle the large multidimensional data sets.

Raw data from flow cytometry were processed and either exported from or analyzed with current versions of FlowJo (Treestar). For clustering methods, uncompensated data are used, but in order to focus our analysis and decrease the size of the data set, we first gated on live T cells and exported this smaller data set for further use. Determination of cell population frequencies and combining expression data after Boolean gating were achieved in FlowJo.

Combinations of simultaneously expressed markers, such as the variety of upregulated inhibitory receptors on a given cell or the polyfunctionality in response to peptide stimulation, were analyzed and visualized with Simplified Presentation of Incredibly Complex Evaluations (SPICE) (295). By combining pie charts and box plots, this user-friendly method allows comprehensive presentation. However, expression of markers is only evaluated to the extent of a cell being positive or negative for any given marker, which does not fully capture the information gathered in flow cytometry.

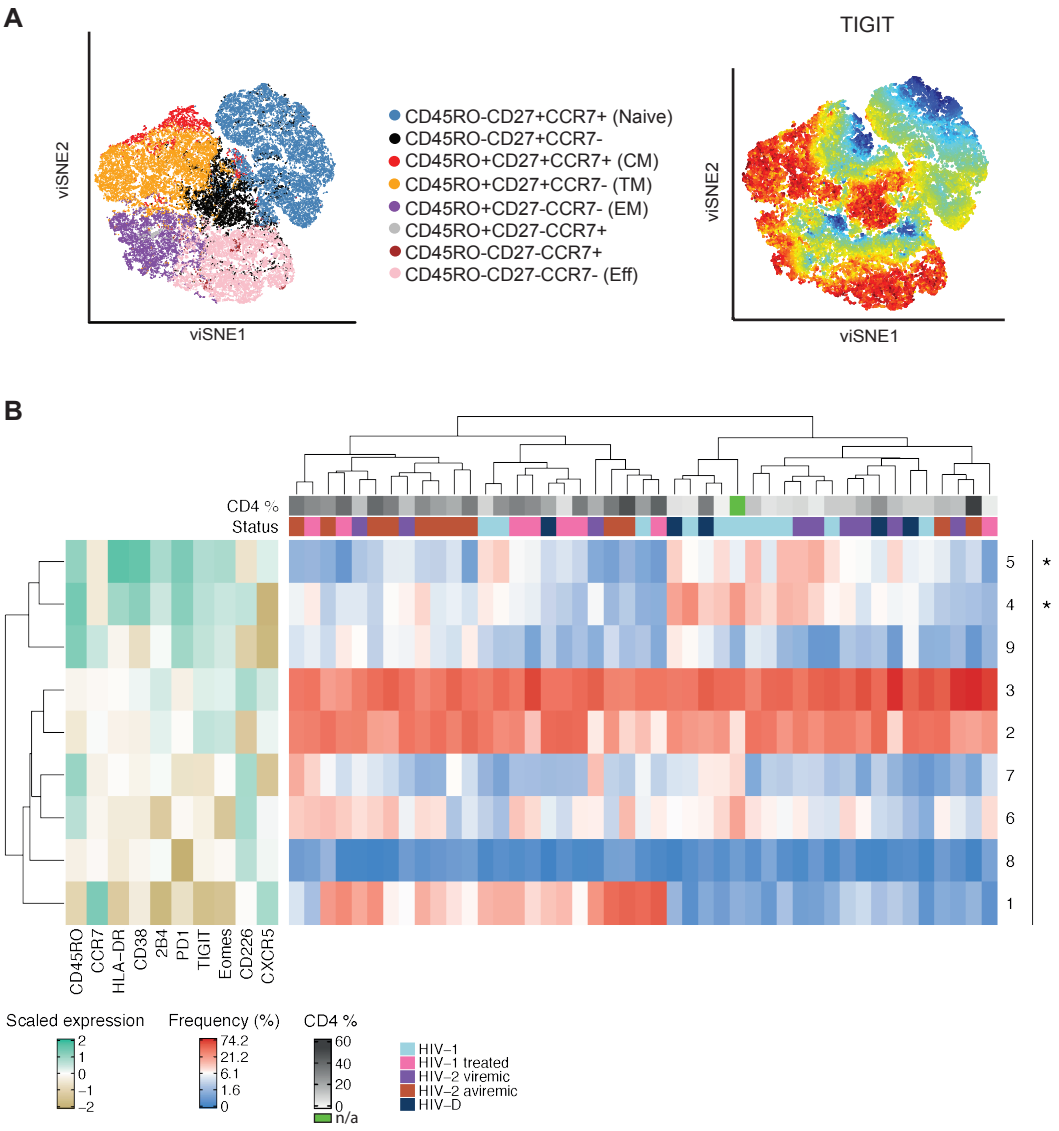

Figure 3. Automated clustering and visualization tools viSNE and FlowSOM. Adapted from **paper I** and **paper III**.

Problems like compensation artefacts (the correction for spectral overlap of fluorescent dyes), scarcity of some cell populations, gradual expression of markers or subjectivity of manual gating can be addressed better by using unsupervised clustering methods (296). Both, viSNE (297) (Fig. 3A) and FlowSOM (298) (Fig. 3B) are visualization tools based on clustering algorithms. They aim to preserve information of the high dimensional data but represent them in two-dimensional plots. ViSNE visualizes cells similar to a scatter plot and can be colored by predetermined cell populations or according to the expression gradient of a single marker. FlowSOM uses self-organizing maps (SOMs) clustering for analysis and a combination of heatmaps and phylogenic trees to visualize how the clusters and patient samples relate to one another.

3.3 MULTIPLEX

For analysis of plasma biomarkers for **paper III**, we used Bio-Plex Multiplex (Bio-Rad) and ProcartaPlex (Thermo Fisher) immunoassays. The approach is similar to flow cytometry in the use of fluorescent probes to identify and quantify different markers. However, instead of attaching probes to cells, magnetic beads are used to capture and quantify biomarkers such as cytokines and chemokines. The magnetic beads are identified by the ratio of two fluorescent dies, creating a two-dimensional matrix of up to 100 distinct beads and thus biomarkers. Antibodies coupled to the surface of these beads capture the biomarker from plasma samples. Subsequently, a second antibody with biotin attached to it is added to the complex. In a last step, the third fluorescent dye is bound to the complex. This third dye allows quantification of the biomarker.

Depending on commercial availability and flexibility, a high number of biomarkers can be simultaneously quantified within a sample, thus minimizing the volume of sample required for analysis. As this project involved blood samples from children, this was particularly helpful. Especially from young children, the volume of available sample can be very low and analyzing a multitude of markers from small sample volumes ensures maximal use from these precious specimens.

3.4 STATISTICAL ANALYSIS

For statistical evaluation of our data sets, we used GraphPad Prism, Stata, R environment and SPICE software. For analysis in SPICE, viSNE and FlowSOM, the integrated statistical tests were used (*e.g.* permutation test and Student´s t test in SPICE). Frequencies of clusters from FlowSOM were also exported and subsequently analyzed in GraphPad Prism.

For comparison of two groups, we used Mann-Whitney U test for unmatched samples and Wilcoxon-matched pairs signed rank test for matched samples (*e.g.* before and after initiation of ART). Comparisons of multiple groups were done with one-way ANOVA and subsequent Kruskal-Wallis non-parametric Dunn´s multiple comparisons test. Correlations were determined with Spearman rank test for the most part. However, analysis of some longitudinal changes and subsequent comparison between the groups in **paper II** required mixed-effects models.

4 RESULTS & DISCUSSION

Systemic immune deterioration and CD8 T cell exhaustion are a prominent feature of HIV infection. Viral and host genetic, host immununological factors, as well as medical interventions influence this process. Here, we analyze markers of immune deterioration with a focus on CD8 T cell exhaustion in treated and untreated HIV-1 infection (**paper I**), the influence of partial protection mediated by HLA-B*57 (**paper II**), aviremic compared to viremic HIV-2 infection (**paper III**) as well as the role of vertical HIV-1 infection and age in children and adolescents (**paper IV**).

4.1 HIV-1-SPECIFIC CD8 T CELL RESPONSES

Persistent antigen exposure is the main driver of T cell exhaustion, thus HIV-specific CD8 T cell responses are affected by immune deterioration to a larger degree than other CD8 T cell populations. However, in their role as antigen-specific T cytotoxic cells in limiting viral persistence, and spread, they might also hold the largest potential for therapeutic intervention.

4.1.1 TIGIT is linked to HIV-1 mediated T cell exhaustion

In **paper I** several groups of PLHIV were studied, including treatment naïve and ART treated individuals experiencing chronic infection, PLHIV during acute infection, as well as elite controllers. To distinguish antigen-dependent exhaustion from systemic exhaustion in HIV, we compared HIV-specific to CMV-specific CD8 T cells in our cohort. CMV is a common regularly controlled chronic human viral infection, besides occasional reactivation like other *Herpesviridae*. We found increased frequency and levels of TIGIT expression on HIV-specific CD8 T cell responses compared to CMV-specific cells in each group (Fig. 4A). Thus, increased TIGIT expression is increased in an antigen-specific manner in HIV infection. In addition, treatment-naïve individuals had higher TIGIT expression on HIV-specific CD8 T cells than ART treated PLHIV or elite controllers (Fig. 4A). As exhaustion is strongly driven by antigen, our results are in line with TIGIT playing a role in HIV-mediated T cell exhaustion. TIGIT was co-expressed with other inhibitory receptors, such as PD-1, 2B4, and CD160, which further confirms expression on exhausted cells.

We then further characterized cells expressing high levels of TIGIT. These cells were more common among HIV-specific compared to CMV-specific CD8 T cell responses within PLHIV. Compared to ART-naïve patients, who displayed a high proportion of CD107a single positive cells, elite controllers and ART-treated HIV infected individuals had more polyfunctional HIV-specific CD8 T cells. The frequency of $TIGIT^{hi}$ HIV-specific CD8 T cells was correlated to the frequency of CD107a single positive cells and inversely correlated to the frequency of polyfunctional cells. This links high levels of TIGIT expression to functionally impaired cell populations. Furthermore, cells with high TIGIT expression more readily displayed a $T\text{-}bet^{dim}/Eomes^{hi}$ transcriptional profile, which is related to exhaustion (218, 299) and might be directly linked to TIGIT expression, as TIGIT can repress T-bet in CD4 T cells (299).

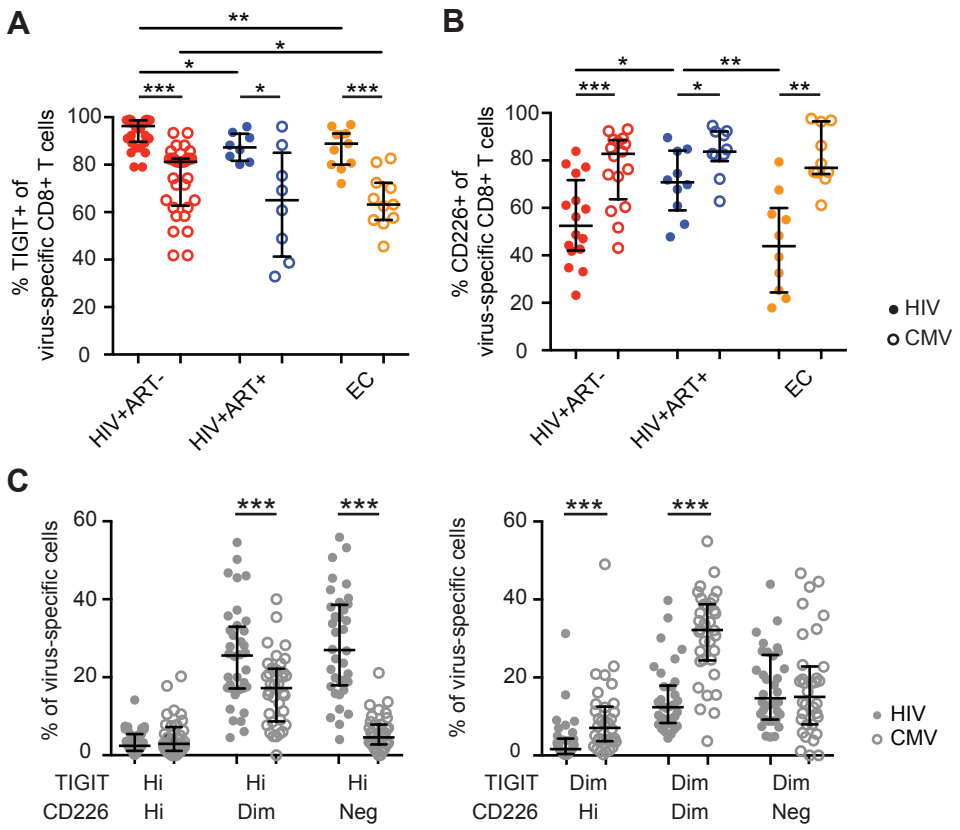

Figure 4. TIGIT and CD226 expression on CMV- and HIV-specific CD8 T cells. Frequency of (**A**) TIGIT positive and (**B**) CD226 positive CD8 T cells specific for HIV or CMV. Cells from treatment naïve (HIV+ART-), ART treated (HIV+ART+) PLHIV and elite controllers (EC) were analyzed. (**C**) CD226 expression on virus-specific CD8 T cells with high (left) and intermediate/diminished (right) levels of TIGIT. Adapted from **paper I**.

TIGIT is part of a signaling network by competing with CD226, a co-stimulator, for binding to PVR. We were able to show that CD226 expression was decreased on HIV-specific cells in comparison to CMV-specific CD8 T cells (Fig. 4B) and that lack of CD226 expression was especially linked to high levels of TIGIT (Fig. 4C). To predict the probability of HIV-specific T cells to encounter the ligand PVR, we analyzed expression on CD4 T cells. We found that circulating and lymph node resident CD4 T cells in PLHIV express higher levels of PVR compared to healthy control subjects. Further, T_{FH} in lymph nodes showed increased frequency and expression levels of PVR compared to memory CD4 T cells in circulation. Expression of PVR was also associated with activation of T cells. T_{FH} were linked to residual replication despite ART (66). Inhibition of HIV-specific CD8 T cell responses in lymph nodes through the TIGIT/CD226/PVR network would thus further impair elimination of lingering HIV replication in treated PLHIV. However, we did not analyze whether the PVR expressing CD4 T cells are infected and data on the influence of HIV on PVR expression are contradictory, reporting downregulation via nef and vpu (300), as well as upregulation via vpr

(301). An additional barrier is the location of T_{FH} inside lymphoid follicles, as CD8 T cells do not enter these sites of HIV replication (302, 303).

4.1.2 TIGIT/PD-1 co-expression is delayed in HLA-B*57 restricted responses

To investigate the influence of the protective allele HLA-B*57:01 on exhaustion of HIV-specific CD8 T cell responses, we investigated a cohort of untreated PLHIV for **paper II**. We compared HIV infected study participants with and without this protective HLA allele and analyzed longitudinal samples, starting after completion of the acute stage and monitoring treatment-free progression. To ensure that differences in progression were not linked to viral dynamics, we confirmed that viral evolution was indeed comparable between the patient groups.

Subtle differences in functionality revealed trends toward higher polyfunctionality in HLA-B*57-restricted CD8 T cell responses, while HIV-specific responses restricted by other HLA alleles tended to have more single expression of either CD107a or IFNγ than their counterparts. In line with waning ability to maintain immune functions over the course of HIV infection, HIV-specific responses from HLA-B*57-negative participants displayed decreased expression of granzyme A over the time of infection. For this study, we selected participants with similar disease progression in both groups. Thus, the increase of CD107a expression among HLA-B*57-restricted CD8 T cell responses reflects the assimilation of clinical characteristics at later time points. In agreement, expression of all inhibitory receptors studied (2B4, CD160, KLRG-1, PD-1 and TIGIT) increased among HLA-B*57-restricted CD8 T cell responses, and 2B4 and KLRG-1 expression increased among responses restricted by other alleles (Fig. 5A).

The main finding of this project was the difference in co-expression of TIGIT and PD-1 (Fig. 5 A-C). In early chronic infection, HLA-B*57-restricted responses had a lower proportion of TIGIT+PD-1+ double positive cells compared to responses restricted by other alleles (Fig. 5 B-C). This co-expression was also associated with disease progression in the form of CD4 T cell depletion and higher viral loads. The stronger correlation with CD4 T cell depletion compared to viral loads might directly or indirectly link exhaustion to the quality of CD4 T cell help rather than viremia. In accordance with progression towards AIDS, the proportion of TIGIT+PD-1+ double positive HLA-B*57-restricted and non-HLA-B*57-restricted CD8 T cells assimilated at later time points.

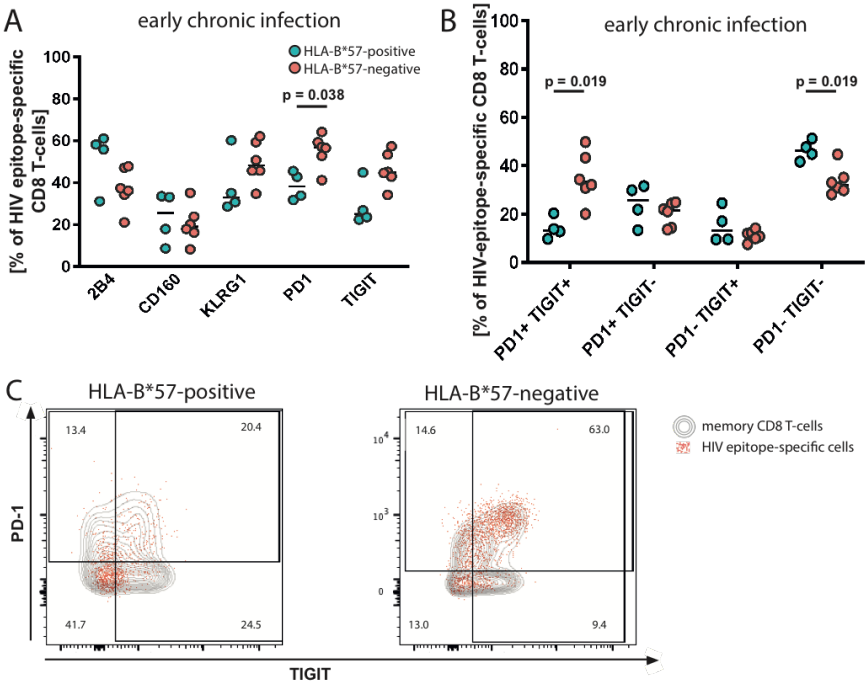

Figure 5. TIGIT/PD-1 co-expression in early chronic infection of PLHIV positive and negative for HLA-B*57:01. Frequency of (**A**) 2B4, CD160, KLRG-1, PD-1, and TIGIT expression and (**B**) PD-1/TIGIT co-expression on HIV-specific CD8 T cells restricted by HLA-B*57 (turquoise) or other alleles (orange). (**C**) Examples of PD-1 and TIGIT co-expression on memory CD8 T cells (grey background) and HIV epitope-specific responses (overlayed red dot plot). Adapted from **paper II**.

4.2 SYSTEMIC IMMUNE DETERIORATION IN HIV-1 AND HIV-2

Independent of antigen-specific immune responses, the immune system of PLHIV is chronically subjected to systemically increased levels of proinflammatory signals and counteracting mechanisms in order to minimize tissue damage. This leads to immune pathogenesis in a more systemic manner than that described for HIV-specific CD8 T cells in the previous section, as a multitude of cell types and anatomic structures are affected.

4.2.1 TIGIT on bulk CD8 T cells

We analyzed bulk CD8 T cells in **paper I** to describe the effects independent of chronic TCR-mediated interactions. We linked TIGIT expression to T cell differentiation and comparing it to PD-1 expression. We saw that TIGIT was more broadly expressed than PD-1 and that most PD-1 positive cells were also expressing TIGIT, but not vice versa. The expression of TIGIT by bulk CD8 T cells followed a hierarchy reminiscent of disease progression, in which the proportions increased from seronegative control subjects and acute infection, to elite controllers, long-term ART treated and was highest in treatment-naïve chronic infection.

We were also able to perform longitudinal analysis of TIGIT expression in patients initiating ART during acute or chronic infection. Follow-up after six months revealed that both the frequency, as well as the expression level of TIGIT increased despite early initiation of ART. Post one year of ART initiation, patients with early initiation had similar TIGIT expression

on bulk CD8 T cells as patients initiating treatment during the chronic stage. This reinforces previous studies showing that prevention of CD8 T cell exhaustion is not improved with early initiation of ART except if treatment is started before seroconversion (304).

The association of TIGIT expression with accumulation of multiple immune checkpoints (PD-1, CD160, 2B4) as well as the T-bet^{dim}/Eomes^{hi} transcription profile was also observed on bulk CD8 T cells. Thus, TIGIT is not only implicated in antigen driven exhaustion, but also systemic immune deterioration of CD8 T cells in HIV-1 infection.

4.2.2 CD8 T cell phenotypes in HIV-2

In HIV-2, the proportion of infected individuals controlling viral replication while staying off treatment is significantly higher, while individuals with viremia experience disease progression comparable to HIV-1 infection. To evaluate the influence of viremia on T cell exhaustion and activation in relation to HIV-1 and HIV-2, a cohort of police staff from Guinea-Bissau (305) regularly screened for HIV infection was analyzed (**paper III**). The cohort allowed for recruitment independent of clinical symptoms and comparison of CD8 T cells between viremic and aviremic HIV-2 infection without ART. In addition, control groups comprised treatment-naïve and treated HIV-1 infected and HIV-seronegative individuals.

Late-differentiated memory CD8 T cells skewed towards an activated and highly exhausted phenotype were more frequent in untreated HIV-1 compared to HIV-2, treated compared to untreated HIV-1, and viremic compared to aviremic HIV-2. Further analysis confirmed that combined expression of activation (CD38 and HLA-DR) and exhaustion (2B4, PD-1 and TIGIT) markers was associated with disease progression in the form of CD4 T cell depletion and VL. We observed the highest frequencies of activated/exhausted cells in viremic HIV-2 infection and that aviremic HIV-2 infection displayed increased T cell pathogenesis in comparison to seronegative individuals. The main causes of T cell exhaustion are viral replication and the consequential inflammation. Our results confirm this link, but also highlights that aviremic HIV-2 infection impacts CD8 T cells despite viral control, potentially through replication below the detection limit.

Subsequently, we analyzed co-expression of activation markers and CD226. Cells without signs of activation but expressing the co-stimulator CD226 were decreased in both aviremic and viremic HIV-2 infection, while activated cells lacking CD226 were increased in both infected groups when compared to seronegative individuals. This suggests a diminished pool of resting CD8 T cells receptive to activation in both HIV-2 infected groups.

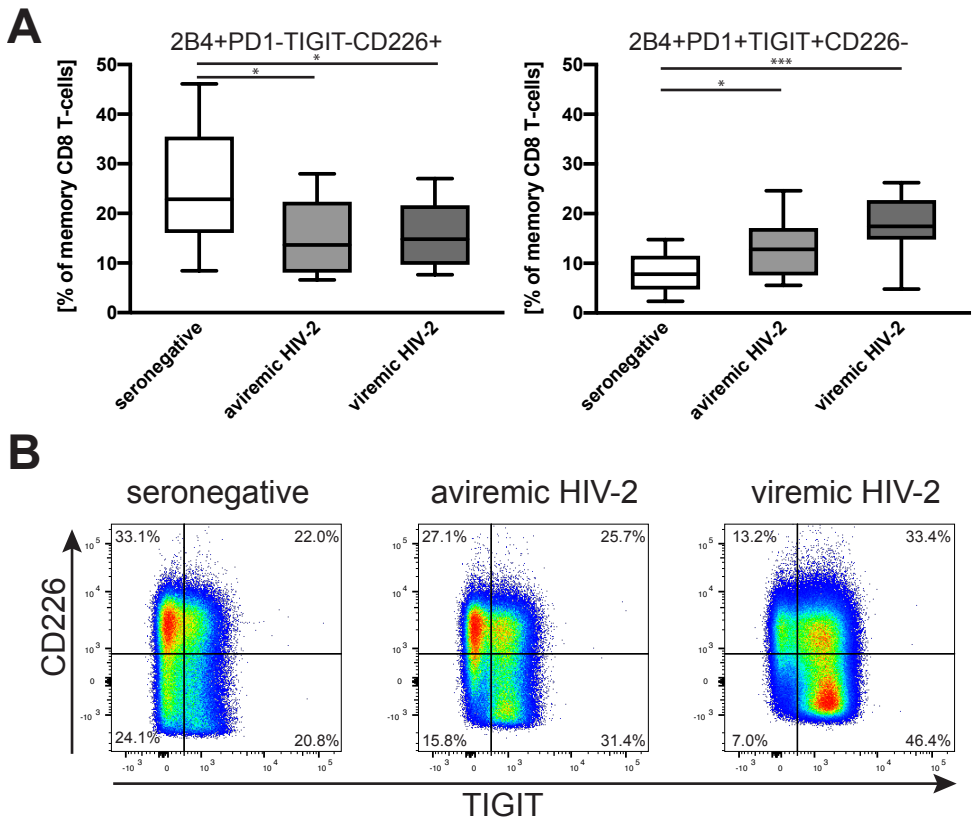

Figure 6. Skewing of CD8 T cells towards an exhausted phenotype in HIV-2 infection despite natural control. (**A**) Frequency of co-stimulation receptive (2B4+PD-1-TIGIT-CD226+) and highly exhausted (2B4+PD-1+TIGIT+CD226-) CD8 T cells, and (**B**) expression patterns of TIGIT and CD226 on CD8 T cells in HIV seronegative control subjects as well as aviremic and viremic HIV-2 infection. Adapted from **paper III**.

Due to the dual role of 2B4 as both an inhibitory and co-stimulatory receptor, we assessed the receptiveness to co-stimulation by combining expression patterns of CD226 and 2B4 with the exhaustion markers PD-1 and TIGIT (Fig. 6A). CD8 T cells expressing 2B4 and CD226, thus potentially receptive to co-stimulation, were decreased and cells expressing 2B4, PD-1 and TIGIT, a highly exhausted population, were increased in aviremic HIV-2 infection compared to seronegative study participants. Lastly, we focused on co-expression of TIGIT and CD226 (Fig. 6B). We found that the frequency of CD226-TIGIT+ cells was increased and CD226+TIGIT- was decreased in both aviremic and viremic HIV-2 when compared to seronegative individuals. This further confirmed the skewed phenotype of bulk CD8 T cells towards exhaustion and reduced co-stimulation receptiveness in HIV-2 infection independent of viremia. As CD8 T cells require CD226 for activation by non-professional APCs (209), the skewing the TIGIT/CD226/PVR network on bulk CD8 T cells could have extensive impact on viral co-infections and malignancies in HIV-2 infection.

4.2.3 Inflammatory markers in pediatric HIV-1 infection

For **paper IV**, we aimed to identify the effects of current ART in pediatric HIV-1 infection on biomarkers associated with immune reconstitution and inflammation. The overall goal of

this project is to identify if there is potential for treatment optimization of HIV infected children in Sweden.

Ligands of CCR5, the main co-receptor in HIV infection, are of interest due to their suppressive impact on HIV infection (154, 157) and their function as proinflammatory chemokines. Of the CCR5 ligands analyzed in this project, MIP-1β and RANTES, had comparable plasma concentrations in vertically HIV infected and seronegative children, and MIP-1α showed a slight trend toward lower concentration in infected children. Both MIP-1α and MIP-1β were decreased in vertically infected adolescents compared to vertically infected children and RANTES was reduced in HIV infected adults compared to vertically infected children. In line with this, the concentrations of MIP-1α and MIP-1β decrease with age in both children and adolescents, while RANTES decreases during adolescence. As we found no significant difference between HIV infected and seronegative children, this suggests higher levels of chemokines in children compared to adults. In addition to their potential to compete with HIV for binding to CCR5, MIP-1α, MIP-1β and RANTES are also proinflammatory chemokines. Successful viral suppression achieved with ART might shift the predominant effects from competition with HIV entry to promoting chronic inflammation. MIP-1β is associated with unsuccessful increase in CD4 T cell counts after ART initiation(160) and MIP-1α affects lipid metabolism (161) and thus cardiovascular comorbidities.

Another biomarker, sTRAIL, has a role in cell death and is thus involved in depletion of both infected and uninfected CD4 T cells in HIV infection (166, 170). The plasma concentration of sTRAIL was the only statistically significant difference between HIV infected and seronegative children in our cohort (Fig. 7 A). Plasma concentrations of sTRAIL were higher in HIV infected children, but decreased with age in infected children and adolescents, which was confirmed with lower concentrations in HIV infected adolescents when compared to children (Fig. 7 A-B).

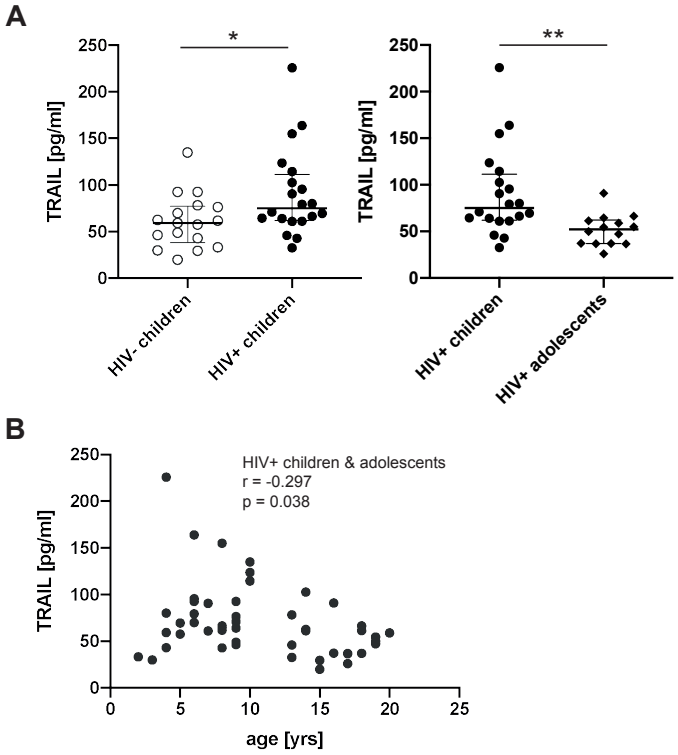

Figure 7. Plasma concentration of sTRAIL in vertically infected children and adolsecents, as well as HIV seronegative children.

TRAIL exists in several forms: membrane bound, soluble and as the non-functional splice variant, TRAILshort. Reduced expression of TRAILshort in elite controllers diminishes inhibition of the functional forms of TRAIL (306), thus links increase in TRAIL to virus control. This suggests an advantage for HIV infected children through increased sTRAIL concentrations. However, the close link to CD4 depletion together with the fact that viremia is suppressed in the majority of our patients, could mean that the pathogenic role of sTRAIL predominates.

The role of cytokines and chemokines is often ambiguous, as they have the potential to increase both, functional immune responses against HIV, as well as the immune activation underlying comorbidities and complications. Factors such as the anatomical and cellular source of the proteins determine which of the two effects outweighs the other.

In contrast to other markers evaluated in this project, sCD14 is the result of microbial translocation, but has only minimal impact on HIV-specific responses. In our cohort, sCD14 was higher in HIV infected children compared to HIV infected adults, but lower than in HIV negative children, though this difference was not statistically significant. This contrasts earlier studies that found increased sCD14 levels in HIV infected children older than 2 years (307). However, it is important to note, that microbial translocation or a “leaky gut” is the physiological norm in infants and gut barrier function is not fully developed until the age of two years (308). Thus interactions between the developing gut barrier and mucosal immune system with HIV and timing of treatment initiation might affect concentrations of CD14 in a durable manner.

Fortunately, vertical infection is very rare in Sweden and most pediatric HIV patients have been infected prior to their arrival to Sweden. However, this results in a large heterogeneity of both patients (regarding ethnicity, age of diagnosis and ART regimen in their country of birth) and virus (*e.g.* higher frequency of HIV clades like HIV-1C that are rare in adult Swedish risk groups) within a small patient cohort. The limitations with regards to size and heterogeneity of our cohort probably contribute to the lack of statistically significant findings in this analysis. However, vertically HIV infected children and adolescents in Sweden are well treated and monitored closely, which is the main reason for the minimal differences between infected and HIV negative children regarding the plasma biomarkers investigated.

5 CONCLUSIONS & PERSPECTIVES

5.1 HIV-SPECIFIC CD8 T CELL RESPONSES

We showed that TIGIT, especially when expressed at high levels or co-expressed with PD-1, has an important role in exhaustion of HIV-specific CD8 T cells (**paper I and II**). High levels of TIGIT as well as TIGIT/PD-1 co-expression correlate with impaired functionality of HIV-specific CD8 T cells and an exhausted transcription factor profile. HIV infection was previously linked degranulation of vesicles devoid of cytokines or cytotoxic molecules and consequential incapability to eliminate HIV infected cells (216). We observed that TIGIT expression was associated with this particular defect. In contrast, elite controllers showed reduced frequency and levels of TIGIT expression, and the protective HLA allele HLA-B*57 can delay TIGIT/PD-1 co-expression. Furthermore, ART can slow down, but not prevent upregulation of TIGIT.

T cell exhaustion is an alternative differentiation path, deviating from effector and memory fates, and is thus detectable in lasting changes to the transcription factor profile. Previous studies linked downregulation of T-bet and upregulation of Eomes, resulting in a T-bet^{dim}/Eomes^{hi} profile, to CD8 T cell exhaustion (218). We were able to show the association of high TIGIT expression on HIV-specific CD8 T cells with T-bet^{dim}/Eomes^{hi} (**paper I**). More recently, early differentiation events towards an exhausted T cell fate have been linked to TOX (219) and TCF-1 (309). The size and maintenance of this progenitor pool might have direct implications in immune deterioration and thus disease progression in HIV. Maintenance of a larger progenitor pool with improved proliferative capacity could maintain a larger frequency of early differentiated immune responses with at least partial polyfunctionality. Alternatively, increased differentiation towards an exhausted fate might suppress other differentiation paths, thus limiting memory and effector T cells. The frequency of TOX and TCF-1 expression among HIV-specific CD8 T cells, especially in treatment naïve PLHIV with different disease progression could give insight into the role of this progenitor pool in HIV infection.

A number of factors influence T cell exhaustion, prominent among those are the quantity and quality of CD4 T cell help and duration and level of antigen exposure. In accordance, we were able to show that successful treatment and thus reduction in antigen treatment can slow down, however not prevent nor fully reverse, CD8 T cell exhaustion. Furthermore, elite controllers, a small group of PLHIV with natural suppression of viral replication and maintained CD4 T cell levels, show reduced frequency and levels of TIGIT expression. Among host genes, HLA alleles are among the strongest contributors to the rate of disease progression (221). In addition to factors like the targeting of specific epitopes, protection by HLA alleles was also linked to the ability to establish new responses targeting emerging viral mutations and the ability to produce IL-2 (214). Here, we showed that the partial protection of HLA-B*57 in the context of HIV infection also extends to a delay in CD8 T cell exhaustion (**paper II**).

As HIV targets CD4 T cells, it creates a pathogenic cycle in which elimination of HIV infected cells does simultaneously curb viral replication, but also diminish CD4 T cell help and thus fuel exhaustion. Checkpoint blockade targeting receptors such as TIGIT and PD-1 could potentially counteract this immunopathogenesis, as theorized in "shock and kill" strategies (279). However, data on PD-1 indicate that blockade is only effective on cells with intermediate, not high expression levels of PD-1 (204, 205), and efficacy of TIGIT blockade requires expression of CD226 (310). Our data indicate that reinvigorating existing and preventing advanced exhaustion of novel HIV-specific responses will be blunted by the frequency of cells expressing high levels of inhibitory receptors as well as skewing of entire signaling networks, as observed in the downregulation of CD226 and simultaneous upregulation of PVR in this context.

Detailed knowledge of exhaustion markers and how their expression patterns are linked to the severity of the deterioration can be utilized in monitoring vaccines and therapies under development. Different vectors, adjuvants and administration strategies might influence not only the development of HIV-specific responses upon vaccination but might also determine their maintenance.

5.2 SYSTEMIC IMMUNE DETERIORATION

We found dysregulation of bulk CD8 T cells in the form of a skewed TIGIT/CD226/PVR network in ART treated HIV, including PLHIV who started ART early (**paper I**) and in natural control of HIV-2 infection (**paper III**). Further impairment of co-stimulation and exacerbation of inhibition in combination with CD8 T cell activation was seen in aviremic HIV-2 infection (paper III). Lastly, progressive upregulation of TIGIT and PD-1 in HLA-B*57 positive PLHIV, but consistently high expression in the absence of HLA-B*57 shows a link between exhaustion and the partial protection mediated by this HLA allele (**paper II**). Together, our results suggest extensive pathogenic changes to the bulk CD8 T cell pool in HIV infection, spanning ART mediated and natural control, as well as HIV types. TIGIT is one of the key players in this deterioration through competition with CD226, synergy with PD-1 and correlation with the expression of other inhibitory receptors.

The dysregulation of CD8 T cells documented in HIV-2 infection (**paper III**) included expression of 2B4 either in concert with CD226 or along with TIGIT and PD-1. The contradictory functions of 2B4 as an inhibitory or co-stimulatory receptor (212) and the potential synergy with CD226 (213) imply functional consequences of this expression pattern. However, these studies regarding 2B4 have been performed on other cell types. Functional studies regarding 2B4 co-expression patterns on CD8 T cells would allow for better interpretation of our results.

The CD8 T cell exhaustion pattern identified in our cohorts supports their biological relevance for underlying complications such as higher risk of cancer, loss of control over chronic infections or reduced protection by vaccinations. The impact on the immune system at large and diminished quality of CD8 T cells in particular will also need to be considered

when attempting immunotherapies to ameliorate said complications. Checkpoint blockade affects novel T cell responses (311), thus efficacy depends on the availability and quality of bulk CD8 T cells, and adoptively transferred CAR T cells depend on the quality of the T cell pool used (312). The skewing of the TIGIT/CD226/PVR network along with the co-expression with other inhibitors implies that additional efforts, such as stimulation of CD226 expression, will be necessary in PLHIV to achieve outcomes comparable to HIV negative patients.

The immunopathogenesis in HIV-2 despite natural control of viral replication is reminiscent of HIV-1 LTNPs and ECs. Maintenance of this viral suppression requires immune activation and might thus be the cause for the limited immune deterioration that still persists in comparison to the seronegative population. However, ECs still have a higher risk of non-AIDS complications and treatment despite viral control has been suggested for this group of PLHIV (313). Similarly, we suggest that treatment of HIV-2 infection despite natural control might curb immune deterioration in this population.

In **paper IV**, we evaluated plasma biomarkers in vertically infected children and adolescents. The quality of medical care in Sweden results in limited differences between HIV infected children in comparison to HIV negative children. A more thorough investigation by focusing on the candidates found in this project might reveal targets for optimization. Alternative forms of the investigated biomarkers (*e.g.* TRAILshort in comparison to sTRAIL), their cellular sources and receptors on key cell types impacted will allow stronger conclusions. To expand our analysis, T cell pathogenesis will be analyzed by flow cytometry. Both functional aspects, such as cytokine production and proliferative capacity, and phenotypic changes, regarding exhaustion marker expression and CD4 and CD8 T cell subsets, will be included in this characterization.

To alleviate the burden of comorbidities in treated PLHIV, several approaches have already entered clinical trials in adult patients (251). However, many of those will not be applicable to children. First and foremost, the reduction of triple combination ART to a regimen of two drugs might lead to even higher complications in children, as they are already at higher risk of virological failure. Compounds such as statins, which lower inflammation via reduction of cholesterol, operate indirectly and their primary effects need to be considered thoroughly. Thus, new candidates for targeted therapies specific to HIV infected children might be necessary to improve treatment outcome in this cohort.

5.3 CONCLUDING REMARKS

Systemic immune deterioration seen in HIV infection is the driver of disease and has implications both directly on HIV/AIDS, but also on numerous non-AIDS complications. Modern immunomodulatory therapies have opened the door to use our knowledge on immune responses in a therapeutic way. While direct use of immunotherapies against HIV is still restricted to preclinical research, the severe side effects accepted in some cancer patients are not considered ethically justified in well treated PLHIV. However, increased risk of

malignancies in PLHIV and expansion of applications of immunotherapies will increase the likelihood for PLHIV to be considered for this type of therapy in one way or another. Detailed knowledge of immune deterioration and exhaustion in HIV might thus guide care for PLHIV in the future.

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