Faculteit Farmaceutische, Biomedische en Diergeneeskundige Wetenschappen

Departement Biomedische Wetenschappen

Exploring biomarkers of the viral reservoir and anti-viral CD8$^+$ T cell responses - towards a functional cure of HIV-1

Exploratie van biomerkers van het virale reservoir en anti-virale CD8 T cell responsen – op weg naar een functionele genezing van HIV-1

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Proefschrift voorgelegd tot het behalen van de graad van

doctor in de biomedische wetenschappen aan de

Universiteit Antwerpen

Promotor Prof. dr. Guido Vanham Antwerpen, February 2020

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Acknowledgements

"A human is not, if he wants to be he has to invent himself." (based loosely on Jean-Paul Sartre)

This philosophical statement is special for me. Although we physically exist, being a human is not given by nature. Life is a continuous journey to invent ourselves. However, current evidence in psychology and cognitive sciences suggests that the "self" is a distortion, an inner narrative and much of our behavior is driven by evolutionary conserved automation. From the perspective of biology, we might be tempted to assume that the existence of the "self" serves the survival of our species. This maybe because it integrates the input from our senses and leads for example to the power of beliefs which link to motivation. In this context, psychologists might add the aspect that a biased or distorted "self" reduces cognitive dissonance hence decreases biological stress. But what is it then then the human and its "self"? Be it as it may but truly fascinating. Fascinating especially from the multiplicity of perspectives. Sciences is a field of technological invention. To be an inventor in human life however it demands a broader perspective. This doctoral thesis is dedicated to cross-domain thinking in sciences. Or how can we invent ourselves as a pluralistic society.

Overall, I feel humbleness to have had this opportunity. Working for the last four years on a doctoral thesis in a field of sciences is a privilege. Nevertheless, all earlier reflection and inspiration aside, it was a struggle. We all know how easy things appeal to us in theory. Be it the manual of our car, a simple recipe of Italian pasta or as a method from scientific publications. In practice there is much more to it. Information and knowledge are two very different things. Sometimes what we call luck makes the difference if a project turns out fruitful. Then there is the thing called human. You and me. Him or her. A doctoral thesis is embedded in a team. Arguments are rarely key to persuasion. Trying to understand the motivation and behavior of others. It might be often far more plain. The automated and egoistic "self". Overcoming the fight against the windmills of our judgements. We all are sitting in Plato's cave.

My first words of gratitude go to Guido Vanham, professor of virology in Antwerp. Over the last four years you have been deeply dedicated to this project. Many thanks for you persistence, scientific dialogue and inspiration. Secondly, Carole Devaux, as the leading PI of the laboratory at the Luxembourg institute of health, has been the initiator, daily supervisor and co-promoter of this project. Many thanks for providing opportunities, several interesting projects to work on and a state of the art research environment. Next, the research fund of Luxembourg (FNR) merits credits for having granted this project over four years. No money no honey.

At this point, I would also like to mention the clinical and scientific collaborators who contributed with collection of biomaterial and scientific expertise. Many thanks also to all patients who have participated in two studies of the presented work.

Furthermore, I am very grateful for the contributions, support and relationship to several colleagues from the laboratories. My best wishes and thanks to Mathieu (“le chien a survécu”), Gilles, Jean-Yves, Quentin and Charlène from Luxembourg. Special thanks go also out to Pieter, Elisabeth and Maartje from the institute of tropical medicine in Antwerp.

Finally, I will not be able nor willing to name all of you who matter to me. Those who are around. My friends and family. You are not the ones who did the job but made it worth for me to do so.

My parents and sister (plus Moe) for being the backbone of my life, in high tides or low tides.

Lastly, I want to express my love and appreciation to Jennita, for being who she is.

Muchas gracias. Te quiero.

LIST OF ABBREVIATIONS

ADCC	Antibody Dependent Cellular Cytotoxicity
AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen Presenting Cell
ART	Anti Retroviral Therapy
BLT	Bone marrow Liver Thymus
bnAbs	broadly neutralizing Antibody
cART	Combined Anti-Retroviral Therapy
CCR5	C-C chemokine receptor 5
cDNA	Complementary DNA
CD	Cluster of Differentiation
CMV	Cytomegalovirus
CNS	Central Nervous System
CRF	Circulating Recombinant Form
CTL	Cytotoxic T lymphocyte
CXCR4	C-X-C chemokine receptor 4
DC	Dendritic Cell
DNA	DesoxyriboNucleic Acid
EC	Elite Control
Env	Envelope
GAG	Group specific antigen
GALT	Gut Associated Lymphoid Tissues
GP120	Glycoprotein 120
GvHD	Graft versus Host Disease
HDACi	Hystone Deacetlyse Inhibitors
HIV	Human Immune deficiency Virus
HLA	Human Leukocyte Antigen
HSC	Hematopoeitic Stem Cell
IgG	Immunglobulin G

IR	Inhibitory Receptor
ISG	Interferon Stimulated Genes
IN	Integrase
KIR	Killer Immunoglobulin like Receptor
LRA	Latency Reversal Agent
LTNP	Long Term Non Progression
LTR	Long Terminal Repeat
MHC	Major Histocompatibility Complex
msRNA	multiply spliced RNA
NAbs	Neutralizing Antibody
Nef	Negative factor
NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B-cells
NRTI	Nucleoside Reverse Transcriptase Inhibitor
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PHA	Phytohemaglutinin
PI	Protease Inhibitor
PIC	Pre Integration Complex
PR	Protease
PrEP	Pre Exposure Prophylaxis
PTC	Post Treatment Control
pVL	plasma Viral Load
qVOA	quantitative Viral Outgrowth Assay
RNA	Ribo Nucleic Acid
RT	Reverse Transcriptase
SCID	Severe Combined Immune Deficiency
SGS	Single Genome Sequencing
SIV	Simian Immune deficiency Virus

STI	Sexually Transmittable Infection
START	Strategic Timing of AntiRetroviral Treatment
Tar	Transactivation Response Element
Tat	Transactivator of Transcription
TCR	T cell receptor
TLR	Toll like receptor
UNAIDS	United Nations program on AIDS
VC	Viral Controller
VIA	Viral Inhibitory Activity
Vif	Virion Infectivity Factor
Vpr	Viral Protein R
Vpu	Viral Protein U
WHO	World Health Organization

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1 GENERAL INTRODUCTION

In 1983 the human immunodeficiency virus (HIV) was identified as the causative agent of the Acquired Immunodeficiency syndrome (AIDS) (1). Whereas the early 1920s are recognized as the period of zoonotic crossover and the emergence of HIV in the human population of West Africa the global spread started much later, presumably in the late 1970s (2). Since then more than 70 million people were infected of which about 35 million have died from HIV-1 related causes. Currently an estimated number of 37.9 million people are living with HIV infection (UNAIDS report 2019). Africa still has the highest burden of HIV infection with a prevalence of 4.1% in adults and about two third of the global HIV-1 infected population living on the continent (Figure 1).

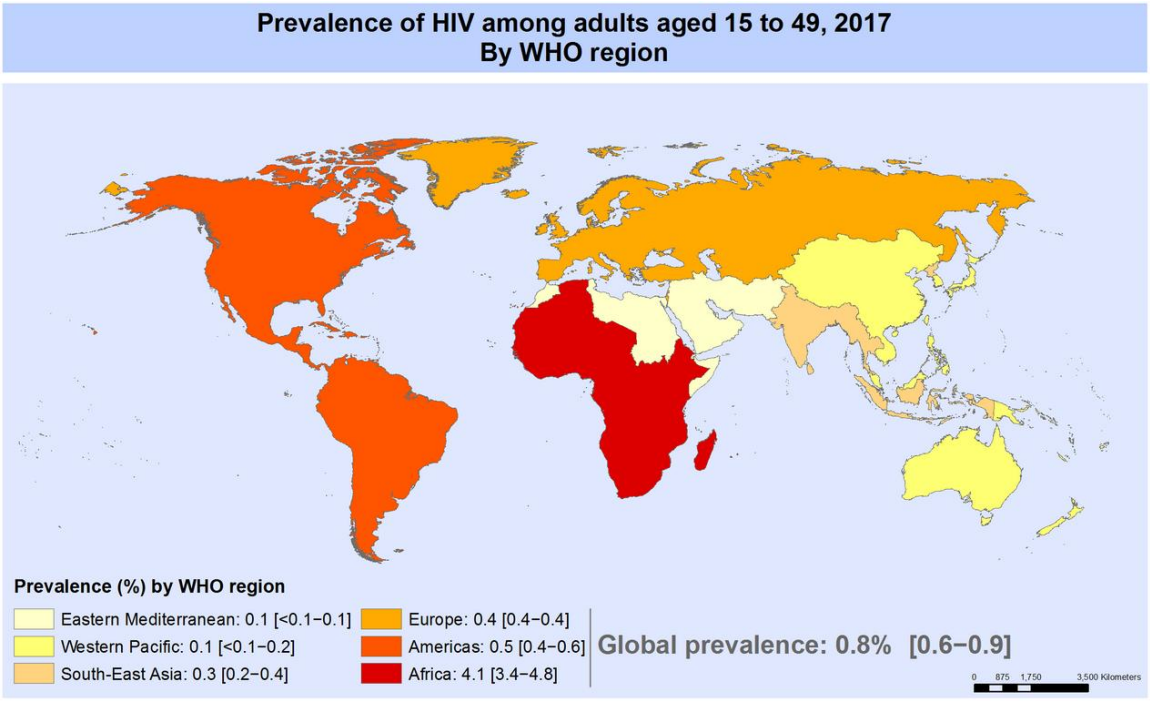


Figure 1. Global prevalence of HIV-1 among adults (age 15-49) in 2017. (source: www.who.int)

The advent of antiretroviral therapy (ART) in the late eighties has tremendously turned the tide in the clinical care of HIV: combined antiretroviral drugs result in long-term suppression of HIV replication and consequently halts the progression to AIDS. Moreover, suppression of viremia has contributed to reduce and stabilize HIV-1 transmission rates (Figure 2). Although ART molecules have further improved (see 2.8) treatment is not curative, because a “viral reservoir” persists (see 2.9) and therefore lifelong therapeutic adherence is mandatory.

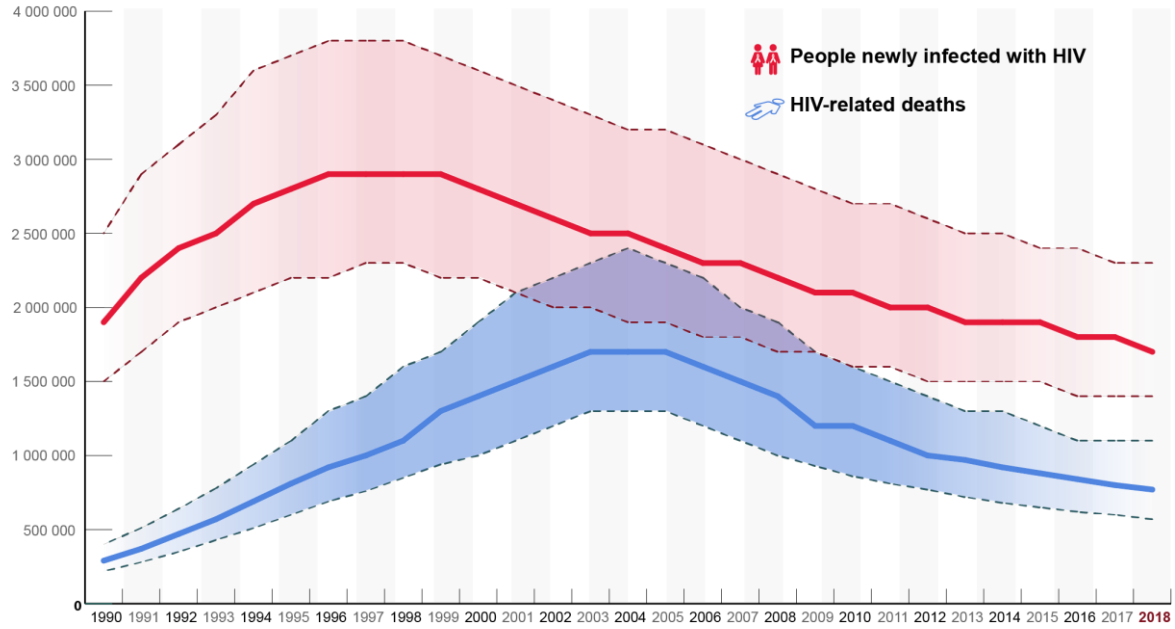


Figure 2. Decline in HIV incidence and mortality over time, Source: UNAIDS/WHO estimates. (https://www.who.int/hiv/data/en/). Red line depicts the trend of global new infections per year. The blue line represents the annual global number of HIV-related deaths. A clear trend of reduction in both parameters is observed over the last 20 years linked to a rise in awareness leading to better prevention of transmission and the introduction of ART therapy.

The UNAIDS program formulated the 90-90-90 goals for 2020 with the long-term target to end AIDS by 2030. These ambitious objectives refer to diagnose 90% of infected individuals, of which 90% with access to treatment of those again 90% being virally suppressed (see Figure 3). The global situation in 2018 of 79-62-53 shows that there is still a lot progress to be made (3), especially for ART coverage. Western and Central Africa (35%), North Africa and the Middle East (24%), and Eastern Europe and Central Asia (28%) are all far below the rest of the world. Treatment scale-up needs be intensely directed to these areas.

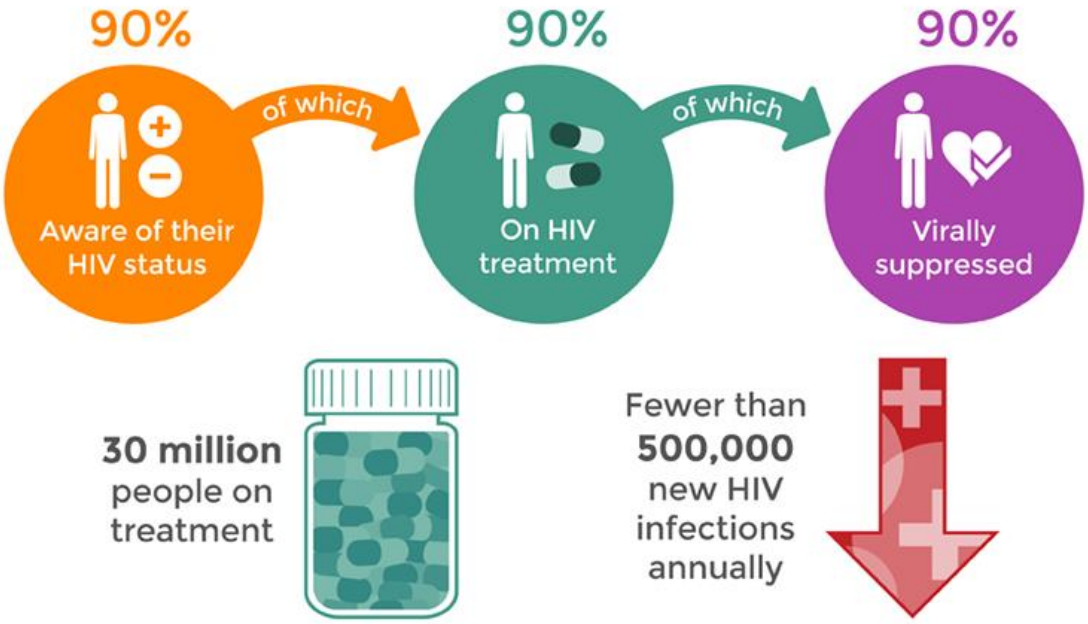


Figure 3. 90-90-90 objectives of UNAIDS (source: from unaids.org) UNAIDS formulated the targets in HIV-1 healthcare for the year 2020. The three objectives are HIV-1 surveillance to increase awareness to 90%, treatment access for 90% of HIV-1 positive individuals and the viral suppression of 90% of patients on treatment.

The Current HIV care model outlines a continuous cascade in place for ideal ART based healthcare. First stands the specific challenge to fill the gap of the lacking awareness of seropositivity (4). Second stands the linkage of testing and treatment services to prevent any delay of therapy initiation (5). Thirdly the adherence to treatment is key which demands measures on psychological, sociological but also the health care supply chain level (6). Lastly, the achievement of viral suppression as a milestone in patient care has to be regularly monitored in order to detect treatment failure early, which might be due to loss of adherence or drug resistance. In addition, viral load testing has to be implemented on a larger scale in resource-limited countries. This step is critical in the long-term strategy of "Treatment as prevention". In that respect the PARTNER and HPTN052 trial have demonstrated that the risk of HIV transmission is effectively zero through unprotected sex with virally suppressed partners (7,8). These results support the in 2017 started "U=U" (undetectable equals untransmittable) campaign promoting the life quality and relief given to patients by successfully monitored treatment.

In Luxembourg, 1176 individuals were estimated to be living with HIV: 1 000 diagnosed patients and 176 (15%, according to the modelling of the European Center for Diseases Control) unaware of their HIV-positive status (Figure 4). As in Belgium the HIV continuum of care for diagnosed patients is high;

of the diagnosed patients, 892 (89%) received routine medical care and 792 (89%) achieved viral suppression. This represents 67% of the estimated people living with HIV for a target of 73%. This exemplifies that the ambitious 2020 targets of UNAIDS are currently not achieved in Luxembourg.

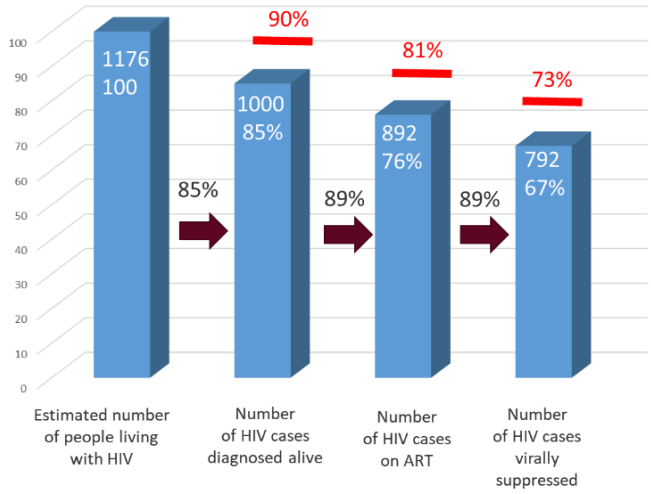


Figure 4. HIV continuum of care in Luxembourg in 2018 (Source: http://sante.public.lu) Public health data of Luxembourg show that only on the level of awareness the 2020 targets are achieved in 2018. In contrast treatment coverage (81%) and viral suppression (73%) need further improvement.

In this introductory chapter, an overview of HIV-1 research is formulated. Over the course, opportunities and innovation for curative strategies will be discussed. Finally, with a focus on anti-viral CD8 + T cell responses during HIV-1 infection the objectives of the present PhD work will be elaborated.

1.1 HIV CLASSIFICATION

Two different types of the virus have been characterized: HIV-1 and HIV-2, both originating from separate zoonotic transfer according to phylogenetic clustering (9,10). HIV-2 originates from sooty mangabey and its prevalence is limited to West Africa mostly (9,11). HIV-1 emerged from chimpanzees and gorillas and is the driver of the global pandemic (12). The difference in epidemiology is linked to the greater infectivity of HIV-1 (13). Although both types share identical transmission routes as well as structural and genetic aspects, they differ in pathogenicity. Clinically HIV-1 infection leads to a much more rapid progression rate to AIDS (14,15). Consequently long-term non-progression (LTNP) is a common phenomenon in HIV-2 infection but rare for HIV-1. The origin of HIV-1 goes back to multiple independent cross-species transmission events leading to the emergences of different lineages, group M, N, O and P. The main (M) group is the driver of the pandemic (16). Lineage M diversifies further into several clades or subtypes which predominate in different regions of the world (17). Subtype B is most prevalent in Europe and America whereas subtype C is predominant in Southern Africa and India (Figure 5). Circulating recombinant forms (CRF) are recombinants between different clades.

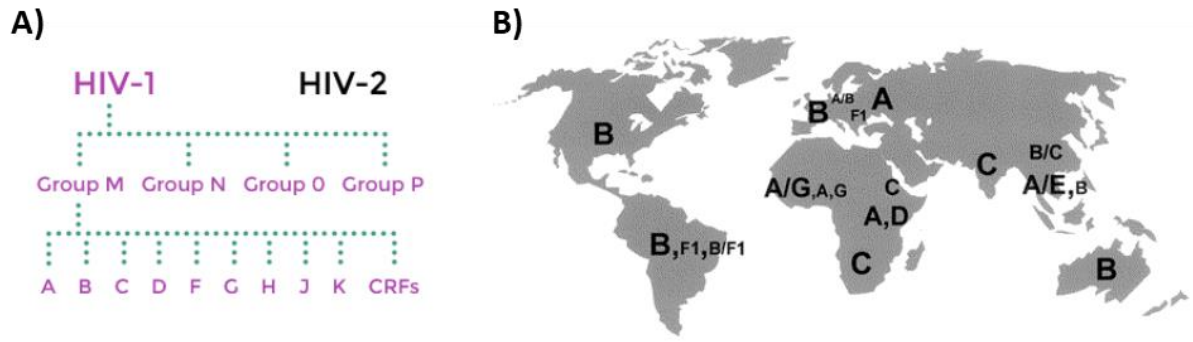


Figure 5. Classifiation and global prevalence of HIV-1 subtype Classification of HIV-1 groups (M, N, O and P) and subtypes of the M group depicted (A). Global geographical distribution of HIV-1 subtypes mapped in (B). (Adapted from www.hie.uw.edu)

1.2 VIRAL STRUCTURE AND PROTEIN FUNCTION

HIV belongs to a subclass of lentiviruses, the family of *Retroviridae*. These are enveloped viruses utilizing reverse transcription of RNA to DNA during replication (18). The basic architecture of a HIV particle consists of three elements: a coat of a lipid bilayer derived from the host cell with viral envelope (Env) proteins, a protein matrix (MA) which surrounds the inner capsid (CA) and a core (nucleocapsid) containing the two identical RNA copies (Figure 6).

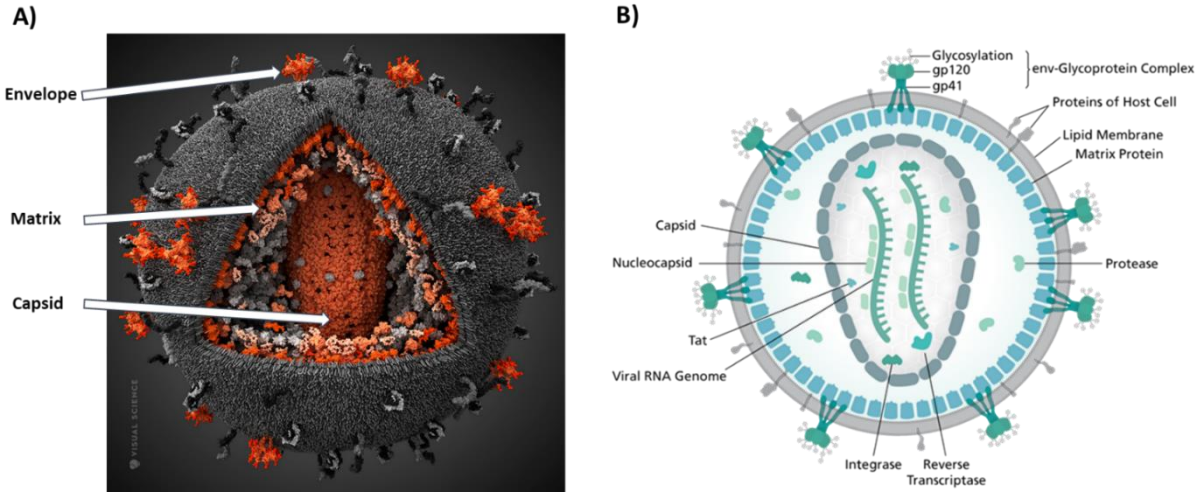


Figure 6. Structure and building blocks of the HIV-1 particle. 3D representation of the HIV-1 particle with the viral proteins in orange and host elements in grey (A) (source: visualscience.ru). Schematic 2D representation of the HIV-1 virion with structural elements annotated (B) (source: Wikipedia, by Thomas Splettstoesser (www.scistyle.com))

The genome of HIV-1, integrated as DNA in the host cell chromosomes, is called a provirus and has a length of approximately 9.8 kilobases. It is flanked by long terminal repeat (LTR) regions which play an

important role during the integration and transcriptional regulation (19). The genome has an intricate architecture, which allows the formation of at least nine different proteins from only 9.8 kilobases of information. These proteins are classified into three categories: major or structural proteins (Gag, Pol and Env); regulatory proteins (Tat and Rev); and accessory proteins (Vif, Vpr, Vpu and Nef). (Figure 7).

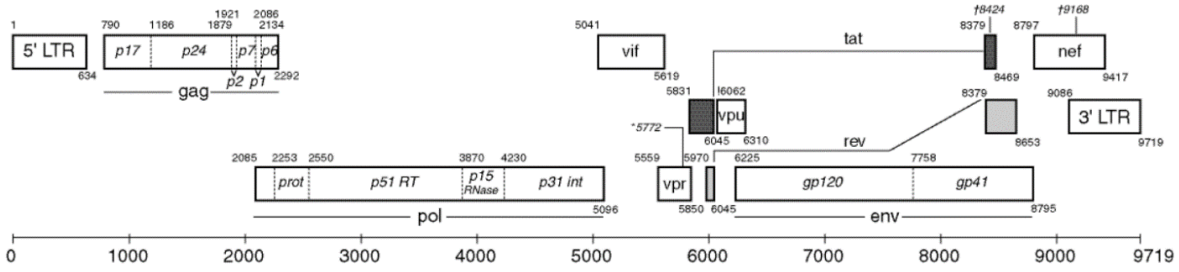


Figure 7. Genomic organization of HIV-1 (adapted from hiv.lanl.gov). Each of the nine open reading frames (ORF) is depicted as a singular rectangle. Gag, Pol and Env are polyproteins, their primary translation products are cleaved into the final proteins (labelled on the ORF) indicated by dashed lines.

The **gag** gene contains the information for the matrix protein (p17) which associates with the inner viral membrane. The nucleocapsid (p7) coats the viral genome and is itself surrounded by the capsid (p24). Another gag-derived protein (p6) is responsible for membrane binding and virus budding (20). The **pol** gene encodes for the three viral enzymes: protease (PR), reverse transcriptase (RT) and integrase (IN). The PR enzyme catalyzes the processing of gag and gag-pol precursor proteins. RT converts the viral RNA into double stranded DNA which is then integrated into the host genome by the IN enzyme (21). The **vif** gene encodes the accessory protein Vif (virion infectivity factor) which functions against the host innate defense such as APOBEC3 (22). The **vpr** gene encodes the accessory protein (viral protein R) which promotes pathogenicity by multiple functions. Among others does it transport the pre-integration complex (PIC) into the nucleus, regulates NF-κB activity and has effects on a variety of genes in the context of cytokine production and antigen presentation (23). The **tat** gene harbors two separately located exons, which translate to the Tat (Trans activator of transcription) protein with an essential regulatory role of viral replication. Its functional domain binds to the HIV transactivation responsive element (TAR) relieving the repression on the LTR region resulting in production of full length viral transcripts (24). The Vpu protein is encoded by **vpu** gene and stands central in the process of virion release where it interferes with the host restriction factor tetherin (22). The **env** gene contains the information for the viral envelop with the surface protein gp120 and the transmembrane part of gp41. The negative factor (**Nef**) accessory protein has multiple functions central for the virulence of HIV-1. Among others Nef enhances viral replication by downregulation of CD4 and coreceptors to prevent superinfection of cells and increase viral budding (25,26). It also

downregulates the HLA class I presentation so that the CD8 + T cell mediated immune response is attenuated, a classical example of immune evasion (27).

1.3 VIRAL REPLICATION AND TRANSCRIPTIONAL REGULATION

The life cycle of HIV-1 starts with adhesion and entry into the host cell (Figure 8). The first step is the binding of the gp120 envelope protein to the CD4-receptor. This interaction leads to a rearrangement of the variable loops of gp120 permitting the interplay with co-receptor CCR5 or CXCR4 (minor coreceptors are CCR2, CCR3 and CXCR6) (28). Subsequently the entry takes place due to a fusion pore created by the gp41 portion of Env with the plasma membrane resulting in delivery of the viral core into the cell plasma. During uncoating of the viral capsid, the viral genome and proteins are released into the cytoplasm. Here the viral RNA genome is reverse transcribed into complementary DNA (cDNA) by the RT enzyme. As a side note, it is noteworthy to mention that the RT enzyme is very error prone and lacks proofreading resulting in 2 x 10^{-5} changes per nucleotide per replication cycle (29,30). This process creates the PIC. Subsequently the Vpr protein enables to transport the PIC into the nucleus of non-dividing cells, a specific feature of lentiviruses (23).

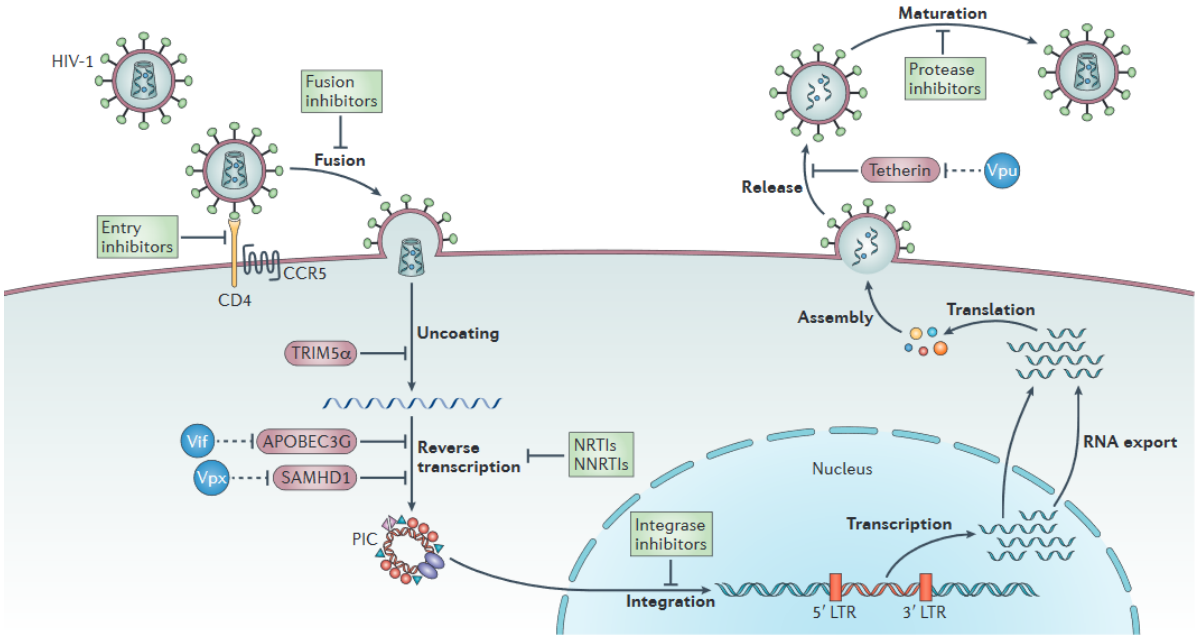


Figure 8. HIV-1 replication cycle (adapted from (31))
The main steps of the viral replication are shown. From initial fusion of viral particle, over the reverse transcription into DNA, to integration and transcription until the release of new viral particles. Drug targets of current ART therapeutics (green boxes) exert effects on multiple steps of the viral life cycle. In addition, cellular restriction factors (red boxes) which interact with viral activity are depicted. These restriction factors themselves can be counteracted by viral proteins (blue circles) an example of intracellular innate immunity.

Once entered in the nucleus the viral integrase enzyme inserts the viral genome into the cellular genome. From here on the provirus is a transcription template tied to the lifetime of the host cell. Viral transcription depends on the presence of nuclear transcription factors. Especially the transcription factors of T cell activation, NfκB and NFAT promote viral transcription. Logically according to status of the infected cell, the provirus can become latent or productive.

The regulatory proteins Tat and Rev control the pattern of viral gene expression. Tat is the activating factor which stimulates elongation of the LTR region into various sliced and unspliced viral transcripts (32). As for all retroviruses, the LTR region acts as the promoter region of viral transcription. The core promoter consists of three element: a triple tandem SP1 binding site, a TAT element and a highly active initiator sequence. All together lead to the binding of the initiation factor TFIID, the first central step of transcription (33). In addition to this core promoter, the replication of HIV-1 relies on enhancer regions. The cellular transcription factors NF-κB and the nuclear factor of activated T cells (NFAT) bind to these motifs. During this early period viral transcripts are spliced into multiple spliced RNA species which are exported from the nucleus and are the templates for Tat, Rev and Nef proteins, the earliest products of viral reproduction (34,35). Tat positively feeds-forward on viral RNA transcription enhancing transcription. Rev plays a role in the export of non-spliced RNA species so that they are available for future viral particles (36). The traffic of all elements and packaging into viral particle is coordinated by Gag and the Gag-Pol polyprotein. The assembled virion buds from the plasma membrane due to Vpu antagonizing the activity of tetherin (37). While the virion buds, its maturation process gets going by the PR enzyme cleaving Gag and the Gag-Pol polyprotein. The virion is now infectious and can enter another host cell and the cycle repeats (38,39).

1.4 TRANSMISSION

The transmission of HIV-1 occurs by the transfer of body fluids. Of note in contrast to other viruses such as influenza, HIV-1 virions are normally not transmitted via saliva (40). The common route is across mucosal surfaces most frequently during sexual intercourse or by direct blood-to-blood transmission of contaminated needles or blood bags. Mother to child transmission, also called vertical transmission, can take place in utero, intrapartum or during breast-feeding (41). The transmission efficacy of HIV is relatively low compared to other sexually transmittable infections (STI) and ranges between 1 in 20 to 1 in 2000 per exposure. This probability depends on factors such as the exposure route and concentration of virus of the infectious source (41). Viral targeting or tropism depends on

the protein interaction for viral attachment and entry. As discussed in the above section, the envelope protein GP120 has an affinity to the CD4 receptor. Consequently, main target population are cells expressing the CD4 protein. These are predominantly CD4 T cells but also monocytes, macrophages and dendritic cells (DC). In addition, the virus needs a co-receptor for fusion. Accordingly, viral species are classified by their usage of the co-receptor CCR5, CXCR4 or both (42).

The early events of HIV-1 transmission *in vivo* remain to be clarified. Mainly because observations during the acute phase of infection typically start when clinical signs of an acute retroviral syndrome manifest in patients. This however commonly presents the earliest several weeks or months after the initial transmission. Keeping this constraint in mind studies the biggest proportion of studies show that CCR5 variants are the initial cofounders of infection (43,44). HIV-1 undergoes rapid and diverse genetic evolution based on its high mutation rates due to the error prone reverse transcription and the quick replication kinetics (45). This is exemplified by the rapid emergence of viral variants, quasispecies, within an individual even though the transmitted founder population is composed of only one or a few strains (46). To date there is no prophylactic vaccine available to prevent infection.

1.5 CLINICAL COURSE OF INFECTION

HIV depletes the CD4 + T lymphocytes overtime consequently leading to a progressive weakening of the immune system. A general classification of the infection divides it into an acute and a chronic phase. Chronic infection can ultimately lead to full blown AIDS. The clinical staging of acute infection starts with the eclipse phase, a short interval of about 10 days post transmission during which the viral replication is limited to the mucosa and none of diagnostic tools can detect HIV-1 systemically. The traceability of HIV-1 RNA in patients' blood by PCR defines the start of the Fiebig stages (47). This period is characterized by a sharp increase in plasma viremia (Figure 9) which mirrors the systemic spread and massive replication especially in the gut associated lymphoid tissues (GALT) where the highest number of CCR5 expressing CD4 + T cells reside (47). It is during this transient peak in viremia that some individuals experience non-specific symptoms resembling the common cold.

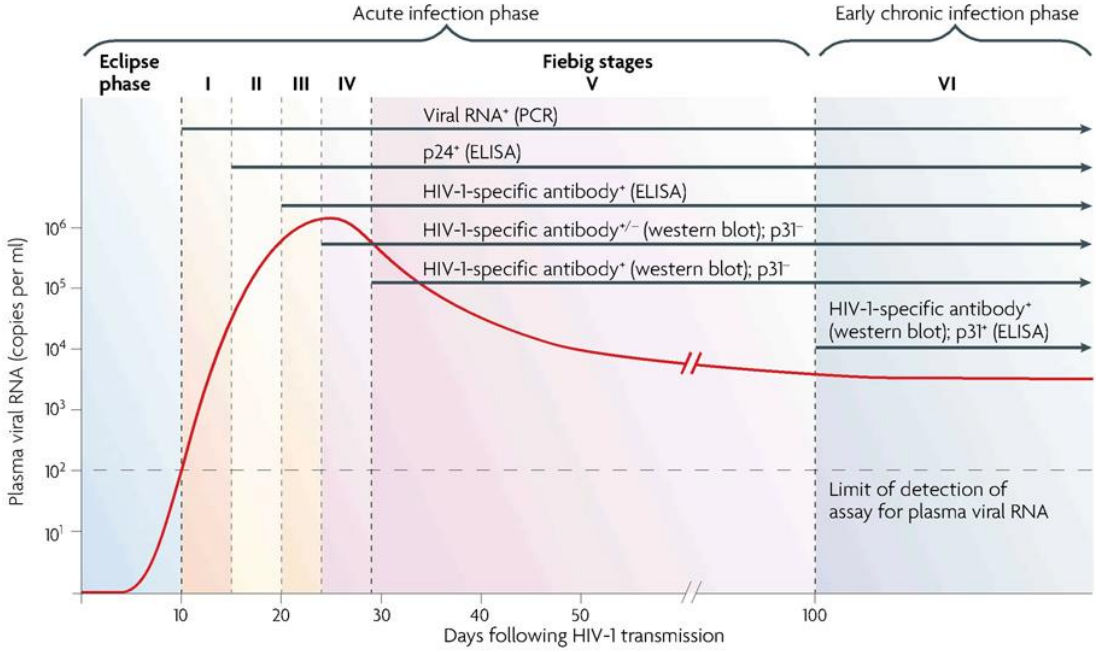


Figure 9. Clinical stages of the acute infection phase of HIV-1 (adapted from (48)). The detectability of HIV-1 infection by different assays subdivides the acute infection into the Fiebig stages. The emplacement of a stable viral set point at about 3-4 months of infection represents the start point of the chronic infection phase in most patients.

In addition to clinical non-specificity, many individuals experience any symptoms at all during the acute infection rendering early diagnosis cumbersome. The sub classification of the Fiebig stages evolves around the diagnostic tests able to detect viral components or the anti-viral immune response. So for example is the seroconversion window, defined as the detectability of anti-HIV-1 antibodies in the plasma, approximately around day 15-25 post acquisition (Fiebig stages III and IV, Figure 9). During this late period of acute infection, the adaptive immune system kicks in. The appearance of HIV-1 specific

CD8 + T cells coincides with declining pVL suggesting a contribution to initial viral control (49,50). In fact the magnitude and rapidity of the CD8 + T cell response during acute infection inversely correlates with the viral set point which is predictive for subsequent disease progression (51,52). With the establishing viral set point, the chronic phase of HIV-1 infection starts (Figure 10).

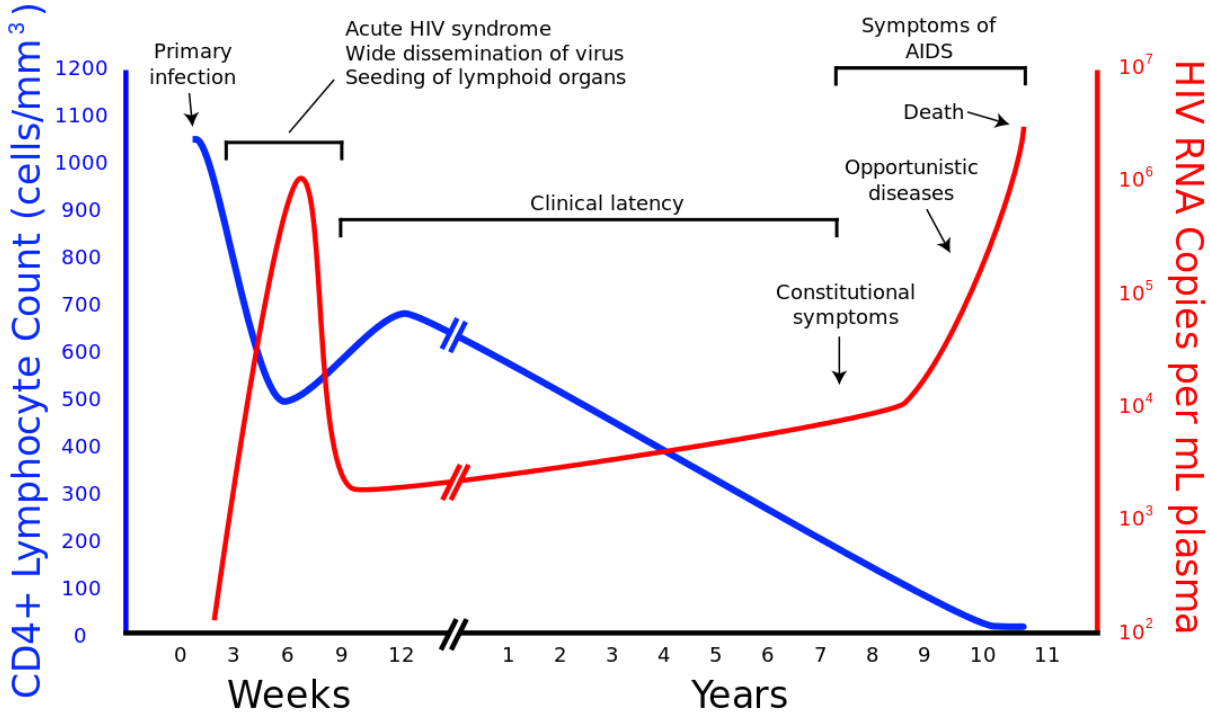


Figure 10. Typical clinical course of untreated HIV-1 infection and progression to AIDS (source: https://commons.wikimedia.org/wiki/File:Hiv-timecourse_copy.svg). The classical progression pattern shows a sharp peak of plasma viremia (red line) during the acute infection followed by a decline to a viral set point. During chronic infection, the plasma viremia slowly and progressively increases over the course of several years. The AIDS syndrome is reached when the absolute count of CD4 + T cells (blue line) sinks below 200 per/ml of blood within about 6-10 years after infection.

This phase lasts from two to ten years and is called the period of clinical latency as most patients experience no or only limited symptoms. Overtime the CD4 + T cells continuously decrease while the viral load increases. This leads in a majority of patients (> 90%) to the development of AIDS within about 10 years. The remaining 10% of patients progress either more rapidly or much slower. Especially the long-term-non-progressors (LTNP), which are about 5% of infected patients, are of much interest in research.

1.6 IMMUNO-PATHOGENESIS

The human immune system divides into two main branches: the innate and adaptive one. This because innate immunity works ad hoc based on recognition of evolutionary conserved pathogen-associated molecular patterns whereas adaptive immunity learns and memorizes specific patterns of recognition acquired through exposure during lifetime. Both systems are strongly interlinked. By nature, innate immunity responds immediately to pathogens and therefore called the first line of defense. This initial inflammatory reaction mounts and orchestrates the pursuing adaptive response. In this context, it is decisive to realize that HIV-1 poses a unique challenge because it directly targets the immune system itself. An appropriate illustration to this complex is the massive depletion of CD4 + T cells during acute infection in the GALT. It leads to a destabilization of the intestinal barrier and consequently to an increase in microbial translocation. This in turn fuels local and systemic inflammatory signals. The enormous replenishment of naïve CD4 + T cells in combination with this inflammatory environment starts a vicious cycle. In the understanding of the HIV-1 infection, it is key that it not only leads to a profound immunodeficiency but also an immense immune activation and chronic inflammation (53). Therefore, detrimental and beneficial effects of inflammation are difficult to delineate as the dichotomy of antiviral immunity and immune-pathogenesis often parallels.

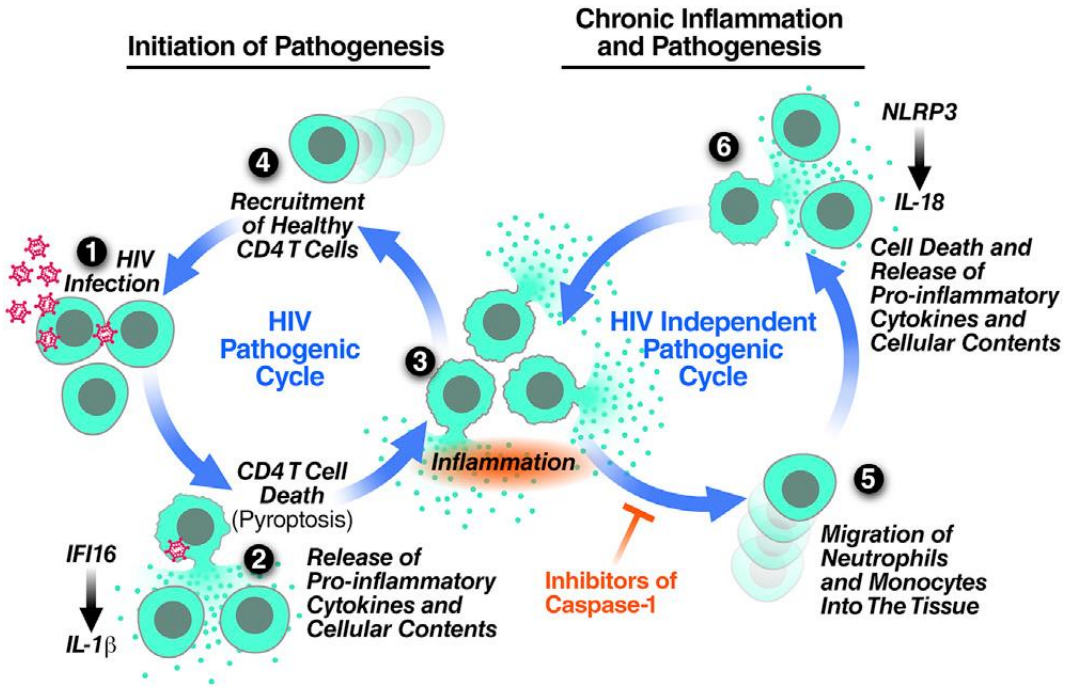


Figure 11. Pyroptosis: a highly inflammatory and common death pathway during direct and indirect killing of CD4 + T cells during HIV-1 infection (adapted from (54)). Direct viral cytopathic effects do not drive the high loss of CD4 + T cells. However intracellular sensing of viral transcripts leads to inflammasome driven highly immunogenic cell death (pyroptosis). In turn, this HIV dependent pathogenic cycle leads to massive bystander effects and can induce HIV independent inflammatory signaling further driving CD4 + T cell loss.

The main localization of viral replication are the lymphoid tissues due to the high density of HIV-susceptible CD4+ cells.The initial peak of viremia is linked to an enormous burst in HIV-1 replication taking place in the GALT which results in depletion of up to 80% of CD4 + T cells within three weeks post infection (55). Although the replication of HIV-1 is understood in detail the mechanisms of CD4 + T cell death are far less clear. Initially it was thought to be a consequence of viral replication thus directly related to productive infection (56). Noteworthy as an enveloped virus, HIV-1 buds from the cell membrane and hence viral replication is not inherently linked to destruction of the host cell (57). One piece of the puzzle lies in the lymphoid microenvironment inducing massive bystander death of neighboring CD4 + T cells by soluble innate immune mediators and cytokines released during direct viral killing (58,59). Recent studies using tissue explant models add the aspect of abortive infection to the picture which induces the inflammasome driven pyroptosis in abortively infected CD4 + T cells (60). With this background, it becomes clear why HIV-1 infection even under treatment has to be considered as a persisting inflammatory disease rather than solely virus driven (Figure 11).

1.7 THE IMMUNE RESPONSE AGAINST HIV-1

The earliest systemic response to HIV-1 is a remarkably high cytokine storm during the first days evoked by innate immunity. This flood of cytokines starts up antiviral gene expression programs such as IFN-I cytokine family exerting activation on the interferon stimulated genes (ISG) resulting for example in upregulation of in-built restriction factors such as APOBEC3 (61). Although non-specific and therefore present during many infections the cytokine storm is much greater than in comparable acute viral syndromes such as hepatitis B and C virus (62). Why that is, is still not well understood. The importance of chronic unabated inflammation as a pathogenic drive in HIV-1 infection is mirrored by the benign outcome of SIV infection in African green monkeys which in contrast to HIV-1 infection in humans, shows early induction of anti-inflammatory factors, such as TGF-beta and IL-10, limiting excessive systemic cytokine signaling (63) .

Moving on in the course of early anti HIV-1 immune response the expansion of the innate cytotoxic natural killer (NK) cell population, especially the $CD56^{dim}$, is observed. Notably later during chronic HIV-1 infection NK cells loose functional characteristic and tend towards a CD56 negative phenotype with reduced IFN-y and antibody dependent cellular cytotoxicity (ADCC) (64–66). Also, it is well documented that HIV-1 evolves mechanisms to reduce the expression of ligands crucial for proper NK cell functioning (67).

Over the course of time, adaptive immunity comes into play. Its arsenal is composed of T and B cells. Both cell types receive instructions directly or indirectly from antigen presenting cells (APC) such as DC via recognition of pathogen derived peptide patterns presented in the context of HLA class I or HLA class II molecules. In combination with supporter cells, CD4 + T helper cells, which deliver crucial costimulatory signals, and shape the cytokine microenvironment an adaptive immune response is orchestrated. Molecular specificity of T cells is given by somatic mutation of T cell receptors during their development leading to a vast array of recognized patterns, called the T cell repertoire. The T cell receptor (TCR) complexes of CD8 + T cells interacts with the HLA-I peptide complexes expressed on all nucleated cells. These peptide motifs, which ensure antigen specificity, are the crucial initiation step to develop CD8 + cytotoxic effector cells, which induce apoptosis by direct cell-to-cell contact or by secretion of proteins into their environment. B cells in contrast are not strictly dependent on HLA mediated peptide presentation and can directly detect conformational epitopes such as polysaccharides or proteins. Once activated and with the proper instructions CD4 + T helper cells and the cytokine environment, B cells can develop to plasma cells as which they secrete specific antibodies circulating in the body fluids.

The antibody response to HIV-1 develops rapidly and antigen-antibody complexes can be found as early as 1 week after detectable viremia however without notable effect on the latter (68). Neutralizing antibodies (NAbs) against the infecting strain appear months later. They drive the evolution of the HIV-1 envelope glycoprotein, the only target on the viral surface antibodies can attach to, reflected by an evolving glycan shield through single amino acid changes (69,70). Since single-cell antibody cloning methods achieved the isolation of broadly neutralizing monoclonal antibodies (bnAb) they got more attention in research. These antibodies have not only neutralization properties but also an enormous effectiveness against diverse circulating strains (breadth). Only a small proportion of HIV-1 infected individuals develops a bnAb response (71,72). Some of the engineered versions of those antibodies (second-generation bNAb) are currently under clinical investigation (such as: NCT02568215, NCT02716675 registered at clinicaltrials.gov) as pre-exposure prophylaxis.

The first CD8 + T cell responses against HIV-1 are detectable during peak viremia of acute infection. At this point, the genetic homogeneity of founder viruses starts to diversify which points to CD8 + T cell mediated selection pressure and subsequent immune evasion (73,74). In addition, the development of an anti-viral CD8 + T cell responses parallels with the initial but temporal control during acute infection (50,75,76). Studies in monkey models further suggest its essential role in maintaining immune control over the SIV virus (77,78). Although high levels of HIV-specific CD8 + T cells persist during

chronic infection, there is no eradication of the virus. On the contrary pVLs remain high and even increase further over time (79–81).

1.8 ANTIRETROVIRAL THERAPY

The introduction of antiretroviral therapy (ART) abolished the automatic death sentence of infected individuals and led to a striking reduction in AIDS related mortality (82,83). Since 1987 when the first drug was introduced into clinic, much progress has been made since mono-therapy lead quickly to drug resistance due to viral mutation (84). Overall, to date 56 antiretroviral drugs that belong to seven classes of therapeutics are marketed in Europe. They are classified according to which step of viral replication they interfere with (85) and include: fusion inhibitors, and entry inhibitors, nucleotide reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), integrase inhibitors and entry inhibitors. In figure 8, you can find the targets of each respective drug class indicated. Future development of antiretrovirals will include improved resistance and cross-resistance profiles such as for new NRTI. Furthermore novel potential targets are on the way such as maturation inhibitors blocking the production of new virions (86). One of the main challenges of cART remains drug resistance. HIV drug resistance accumulates as virological failure persists and necessitates a switch to a second-line regimen therapy such as PI. The development of acquired drug resistance is stabilizing in high income countries as the access to high mutational barrier drugs, such as the integrase inhibitors has increased. However, acquired drug resistance is high worldwide: almost half of HIV-infected patients in sub-Saharan Africa failing tenofovir disoproxil fumarate (TDF)-based cART have resistance to all three antiretroviral drugs (87). Transmission of drug-resistant HIV strains is therefore rising and associated with an increased risk of virological failure in a number of resource-limited countries. As a consequence, this poses a great risk to the ART based healthcare (88) and has been recognized as a threat to the HIV/AIDS 90-90-90 targets.

Over the years, more molecules from the class of nucleoside reverse transcriptase inhibitors (NRTI) were developed and used in mono- or bi-therapy regimens. In 1996, the effectiveness of triple-drug therapy was reported. Since then tri-therapy, also called combined antiretroviral therapy (cART), has been recommended by the WHO treatment guidelines. During the last two decades non-nucleoside reverse transcriptase (RT) inhibitor (NNRTI)-based triple-combination therapy has been the predominant first-line treatment in combination with NRTIs including lamivudine (3TC), zidovudine (AZT), or stavudine (D4T). The thymidine analogs were further replaced by the nucleotide reverse

transcriptase inhibitor tenofovir (TDF) (89). Integrase strand transfer inhibitors (INSTIs) have emerged as the new recommended first-line treatments in 2016, due to a favorable safety profile with fewer adverse events, low rates of virological failure and a higher genetic barrier, especially for dolutegravir. In most cases cART succeeds in suppressing viremia to below 20 copies per ml of plasma (i.e. the current threshold of standard quantification) (90). Co-formulations allow a once daily single tablet regimens, which eases intake and improves therapeutic adherence (91,92). Interestingly recent literature substantiates that two-drug therapies for initial and maintenance therapy are comparably effective and of potential interest because of improved tolerability, less toxicity, simpler regimens and cost reduction (93). The condition is that at least one of the drug has a relatively high barrier to resistance in order to preclude virological failure. In addition the advances in long-acting ART molecules, administrated with injection weekly or monthly will help to improve adherence especially in developmental countries where the reach out to patients is highly challenging (94,95). Furthermore, combination of two antiretroviral drugs was recently approved for pre-exposure prophylaxis (PrEP).

Despite its enormous success, there are other considerable challenges and shortcomings in the ART based healthcare. This concerns the need for strict life-long adherence with the possibility of drug toxicity and drug drug interactions (96). Furthermore, we face an increasing number of ageing ART patients with higher incidence of age-related co-morbidities due to increased low grade inflammation during therapy (97). Whether residual viremia is responsible for heightened immune activation during ART is not fully clear but possibly plays into this (98,99). Consequently, new approaches to complement ART based therapy are needed.

1.9 THE VIRAL RESERVOIR

In the 1990s, ART was believed to lead within approximately 3 years of continuous suppression to the full clearance of virus from the body based on the decay of HIV observed in the blood. However, this presumption turned out to be over-simplified: despite highly effective in suppressing viral replication cART is not curative. Patients interrupting treatment rapidly rebound and quickly reach a pVL similar to their viral set point, because HIV-1 persists as a viral reservoir in various tissues, refractory to the immune response or cART (100,101).

The viral reservoir defines all cellular entities or anatomical compartments in which a replication competent virus stably persists. The two vital elements of this definition are first, the aspect of replication competence which implies the capacity to give rise to fully infectious viral progeny and

secondly the element of stability, which refers to more durable kinetics than actively replicating virus. In addition, we can deduce that a reservoir can be either the pure presence of virions in an anatomical compartment or cell(s) containing replication competent and inducible pro-virus. The localization of both types of reservoirs are diverse and include lymphoid tissues, gastrointestinal tract, central nervous system, genital tract, semen, brain and lung (102).

The key characteristic of HIV-1 reservoir are:

- Its **rapid installation** within days upon infection over multiple sites of the lymphatic system (103–107).
- Its **heterogeneity** in terms of where it hides. Recent literature highlights the heterogeneous nature of cells containing replication competent provirus, be it naïve CD4 + T cells, resting CD4 + T cells, T follicular helper cells in lymphoid germinal centers or macrophages (108–110). This is underlined by the fact that rebound of genetically identical virus emerges from diverse reservoirs of various cellular and anatomical compartments (111). Still, the central memory CD4 + T cells are thought to be the most important cellular reservoirs during ART (112).
- Its **stability**: The decay of the HIV-1 reservoirs under treatment is slow and an estimated duration of at least sixty years of continuous cART would be necessary to clear all infected cells (113).

Three main factors were identified to drive long-term persistence during ART: low-level viral replication, proliferation or clonal expansion of existing cellular reservoirs and viral latency. The continuous viral replication during ART is controversial. Whereas some therapy intensification studies argued in favor of this hypothesis, the absence of viral sequence evolution during therapy argues against it (114–116). Clonal expansion or proliferation of memory T cells containing HIV-1 was identified as a crucial factor of reservoir persistence. Especially the observation of identical viral sequences in CD4 + memory T cells with high proliferative abilities is telling (117,118). Most likely physiological homeostatic proliferation of memory T cells but also the viral integration into host genes associated with cellular growth are underlying factors (119,120).

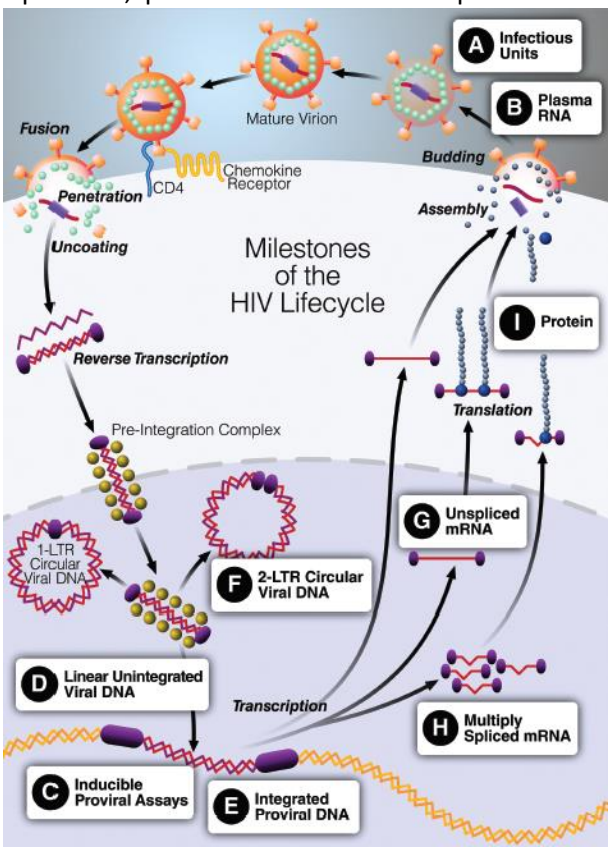


Figure 12. Different stages of HIV-1 life cycle targeted by HIV-1 reservoir assays (adapted from (123). Biomarkers of the viral reservoir include infectious units in plasma (A); plasma viral RNA or antigens (B); inducible proviral assays (C); linear unintegrated viral DNA (D); integrated proviral DNA (E); 2-LTR (long terminal repeat) circular viral DNA (F); unspliced viral messenger RNA (mRNA) (G); multiply spliced viral mRNA (H); viral protein (I).

Taken together the long life span and clonal prolfieration of memory subsets fosters long-term reservoir stability.

HIV-1 latency describes a nonproductive state of infection.We can distinguish pre-integration and post-integration latency (121). The first occurs when integration fails. Instead, the viral DNA auto assembles to 1-LTR or 2-LTR circular strands (Figure 12). These DNA entities can be detected temporarily but no further replication is possible. In contrast, post-integration latency describes the provirus in the absence of viral replication. Consequently, neither drugs nor the immune system can exert any effect on it. To what extent viral transcription is lacking remains controversial as even resting CD4 + T cells show evidence of transcriptional initiation (122). In conclusion, viral latency is clearly the main barrier to reservoir eradication in the presence of potent ART molecules.

1.10 ESTABLISHMENT AND MAINTENANCE OF VIRAL LATENCY

Productive infection of HIV-1 primarily occurs in activated CD4 + T cells (124,125). Resting CD4 + T cells in contrast show obstacles to infection (126). Interestingly though populations with resting memory characteristics are a major and stable reservoir in CD4 + T cells (127). Therefore, the most accepted scenario postulates that very few productively infected cells can revert from an activated to a resting state during memory development. During this transition levels of NF-κB and pTEFB decrease as well as silencing of the HIV genome occurs indicating some mechanisms of how latency is established in a first place (128). However, exact molecular mechanisms are not understood in detail.

Attempts to decipher how viral latency is maintained over time mostly indicate transcriptional blockade at various levels (32,129). There is conflicting data, which of those is the decisive factor. Some studies highlight epigenetic modifications whereas others find no evidence for that (130–133). Furthermore, the cellular resting state of the principal host cells of latent virus suggests low levels of transcription initiation factors (NF-κB and NFAT) is involved in muting viral expression (134,135). The overrepresentation of reservoirs in resting CD4 + T cells supports this notion. Inhibition of viral transcription can also result from interference by active cellular genes neighboring the provirus resulting in “read through” transcripts preventing the effective binding of initiation factors to the viral promoter (136,137). In addition, it has been shown that the RNA polymerase might interrupt after the trans-activation response (TAR) region, leading to an accumulation of short TAR transcripts. These have been detected in cART patients in vivo and suggest to identify inhibition of elongation (138). If these mechanisms also control latency during natural control of HIV-1 remains to be investigated.

1.11 MEASURING THE VIRAL RESERVOIR

The size of the latent viral reservoir is still not precisely determined. The extreme scarcity of latently infected cells in combination with the low amount of intact proviruses makes only 1 in $1x10^6$ – $1x10^9$ CD4 + T cells potential sources for viral rebound (139). Therefore, a high sensitivity (detection of rare positives against a high background of negative cells) as well as specificity (certainty that rare events are true positives) is needed. Furthermore, information on the replication competent and inducible fraction only allow a potentially good prediction for viral rebound *in vivo*. What follows is a brief discussion of the most frequently used assays.

HIV-1 DNA:

Levels of total HIV-1 DNA are assessed using PCR probes targeting conserved viral regions such as gag or LTR. If run in parallel with β-globin or albumin it allows to scale the HIV-1 copy numbers against the amount of cells assayed (140). However PCR based technics enormously overestimate the viral reservoir by about 10-100 fold (depending on the status of patient). This because the vast amount of sequences quantified are not replication competent (141). Another aspect to consider is the viremic status of the sample. During high viremia, hence considerable levels of productive infection, measuring integrated DNA only can be necessary because unintegrated DNA can make up the majority of total DNA potentially confounding results when it comes to reservoir assessment (142). In contrast, the impact of nonintegrated DNA on readouts of HIV-1 DNA is negligible during suppressed viremia. In spite of all limitation, PCR based assays are highly sensitive, established in most labs with comparably low costs and low amounts of biological material needed. Furthermore, a recent study stresses that the pool of integrated and total HIV-1 DNA are suitable surrogate markers for disease progression and time to viral rebound during ART (143).

HIV-1 RNA:

Viral RNA can be quantified by PCR as total amounts, as spliced versus unspliced entities or as the full spectrum of RNA species. When RNA is measured in plasma it can give information on the exact copy number of virion-associated or free viral RNA. Conversely measuring cell associated RNA (CA-RNA) allows to determine the transcriptional activity within a cell thus information on the status of viral reservoirs but not the total size (123).

Full-length single genome sequencing (SGS):

To better estimate the integrity of proviral reservoirs, sequencing can be performed. Genomic DNA is extracted full-length viral transcripts are amplified and subsequently sequenced either by Sanger or next-generation method. Results have indicated the enormous overestimation by PCR methods but also a significant fraction of intact and replication competent provirus leading to a much more precise estimation of true reservoirs (139,144). However, sequencing is laborious and costly. In addition, it cannot capture permanently silenced reservoirs thus does slightly overestimate the true clinical relevant fraction.

Tat-Rev limiting dilution assay (TILDA):

A cell culture based assay measuring the tat/rev multiply spliced RNA (msRNA) upon maximal activation of purified CD4 + T cells in only 12 hours. It allows to calculate the frequency of cells with inducible processive RNA transcripts (145). A limitation is that although tat/rev msRNA gives a good measure of advanced stages of viral replication (see Figure 13) it does not reflect full viral production of functional virion and release from cells. Consequently, it only partially maps the real reservoir albeit more closely than PCR assays.

Quantitative viral outgrowth assay (qVOA):

The qVOA is regarded as gold standard for the detection of the frequency of resting CD4 + T cells harboring replication competent, inducible reservoirs. Purified resting cells (CD3+CD4+ and CD69-CD25-HLA-DR-) are plated in dilutions and activated by phytohemaglutinin (PHA) over 14 days. Recent literature emphasizes the need of several rounds of stimulation to minimize potential underestimation of reservoirs (144).

In conclusion, the search and characterization of HIV-1 reservoirs is clearly challenging. Consequently, cellular biomarkers for the HIV-1 reservoir could accelerate the understanding and characterization of it. In this context different surface markers have been proposed (CXCR3, CD2, CD30, CD32,PD-1, CTLA-4, TIGIT, LAG-3) to pinpoint subsets that preferentially harbor proviral DNA but studies have not converged (146–150). These divergent findings could be rooted in patient-to-patient heterogeneity making a single marker improbable. Especially the controversially debated CD32 remains to be thoroughly characterized in reservoirs of peripheral tissues.

1.12 MODELS TO STUDY HIV-1 LATENCY

Cell line models of HIV-1 are highly valuable to study molecular mechanisms of latency. But they do not faithfully represent latency in natural primary host cells because cell lines are highly mitogenic and therefore very different from *in vivo* reservoirs when it comes to metabolic and transcriptional status (151,152). Still, breakthrough findings for example the identification of first latency reversal agents (LRA), such as histone deacetylase inhibitors (HDACi), go back to cell line models (153). Primary cell models of latency are numerous and come with the advantage of being closer to the status of latency *in vivo* concerning cellular activation state (reviewed in (152)). They have contributed especially in the area of screening for new LRAs but also for potential biomarkers of infected cells (154–156).

Due to the absence of entry receptors as well as post-entry blocks murine cells are not able to be infected by HIV-1 (157). To this end, most *in vivo* studies from the beginning of the HIV-1 pandemic on go back to SIV models. The advantages are at hand, a very similar anatomy, similar reservoir characteristics, high in sample size and a fully functional immune response. Some of the breakthrough findings of the last years include the description of viral reservoirs in CD4+ follicular T helper cells and testing of therapeutic vaccination strategies aiming at latency reversal and T cell vaccination in parallel (158,159). However, SIV remains a different virus, more related to HIV-2 than HIV-1 exemplified by the existence of the Vpx protein which doesn't exist in HIV-1 (160,161). In addition, monkey models are very resource costly and even more problematic from an ethical point of view than other animal models.

Over the last two decades different humanized mouse models of HIV-1 came into practice. As the name suggests these are mice, strains engrafted with human immune cells. General advantages are the comparably easy handling and the recapitulation of key aspects of HIV-1 infection over multiple tissue sites. In the next paragraph, we will discuss the main models concerning their strength and drawbacks with particular focus on studies of HIV-1 latency.

First studies used the so called *hu-PBL-SCID* model, which translates to the injection of human peripheral blood lymphocytes injected in to severe combined immunodeficiency (SCID) mice (162). Although engraftment dramatically enhanced since used in the NOD SCID gamma mouse (NSG), which also lacks murine NK cells, this strategy leads to a rapid development of graft-versus-host disease (GvHD), limiting the duration of experimentation to about one month (163). While the xenograft situation has also an advantage to study viral reactivation on the short run, long-term studies on latency in this model are not possible (164).

In the *Hu-HSC* approach, mice are subjected to sub lethal irradiation regimens or chemotherapeutic agents to open up bone marrow niches for the engraftment with human hematopoietic stem cells (HSC). If applied to newborn NSG mice it leads to a robust engraftment of multiple organs with lymphocytes as CD4 + T cells and comparably long term stability of about 6 months post engraftment (165,166). The clear limitation of this model is that lymphocytes are not educated in a human thymus but with the murine MHC system which leads to no detectable specific T cell responses.

In this regard, much enthusiasm derived from the *Bone marrow-liver-thymus (BLT)* model. Due to implantation of human thymus and liver tissue as well as HSC anti-viral T cell responses could been detected suggesting full recapitulation of T cell education (167). The downside is the rather rapid development GvHD at about 3-4 months post engraftment.

By nature a proper representation of HIV-1 latency demands a long experimentation window. Animals have to be engrafted, infected and subsequently treated for sufficient long periods in order to recapitulate key aspects of latency. BLT and Hu-HSC models have both been used to establish suppressed viremia using ART and resting human CD4 + T cells have been shown to contain re-inducible HIV-1 (168,169). Albeit all advances humanized mice remain xenograft models and have considerable limitations as such (170). First GvHD restricts the experimental timeline. Consequently, long-term mechanisms of reservoir maintenance are not reproducible. Secondly, the xenograft environment imposes an inflammatory context which can spontaneously reactivate virus in a way not seen in patients (171).

Collectively, available models harbor considerable opportunities if thoroughly monitored for GvHD in studying HIV-1 latency. Further advances in experimental procedures will help to generate humanized mice with even longer life span.

1.13 HIV CURE

When the HIV-1 virus was identified as the causative agent of the AIDS syndrome, the hopes were high to have quickly a vaccination formula at our disposal inducing prophylactic protection. However, it became apparent that protective immunity is not easily induced due to HIV-1 rapid genetic divergence. Till this day, only the RV144 trial provided modest protection against HIV-1 acquisition attributed to the development of non-neutralizing IgG against the V1/V2 region of the viral envelope as well as antibody-dependent cellular cytotoxicity (172). In contrast to most other Env-based efficacy trails the STEP study was designed to elicit cellular immune responses. Although the chosen adenovirus vector containing clade B Gag/Pol/Nef was immunogenic and well tolerated, an interim analysis revealed alarming increase of HIV-1 acquisition in the treatment arm. The trial was immediately stopped and even though it failed, the follow up analysis revealed a selective pressure on the infecting virus, presenting the first evidence from vaccine induced T cell responses in HIV-1 (173). A factor involved in the detrimental outcome is most probably the pre-existing humoral immunity to adenovirus (174). The mechanistic basis for this process remains to be understood.

In 2009, HIV-1 CURE became reality. Timothy Brown, the Berlin patient, was the first person considered cured to this day. He underwent two rounds of full body irradiation followed by bone marrow transplantation due to an acute and recurrent myeloid leukemia. In his case, the transplant donor had a rare homozygous CCR5Δ32 genotype which renders his cells resistant to infection by CCR5 trophic HIV-1 strains (175). Today the extensive conditioning in combination with the resistant genotype are regarded as conjoint contributors to this success (176). Instinctively clear is the fact that this life threatening intervention was never viable nor ethical to scale up to a substantial numbers of HIV-1 infected patients, remaining restricted to those who need lethal irradiation and hematopoietic stem cell transplantations for leukemia. Nevertheless, truly did it inspire and create new hopes that a CURE is reachable. Just recently, even though multiple times attempted and failed in other cases, a second patient cured by stem cell transplantation was reported, and so called the London patient, emphasizing that HIV cure is reachable(177). By definition, a full eradication of HIV-1 from the body is called as sterilizing CURE whereas long-term remission without ART but still detectable HIV-1 reservoirs is termed a remission or “functional CURE”.

Much excitement for a functional CURE was derived from observations of secondary or post treatment control (PTC) (178–180). The VISCONTI trial observed sustained PTC for years after ART cessation (180). Although patients share the feature of low cell associated HIV-1 DNA with natural controllers, other

characteristics such as the CD8 + T cell response or the enrichment of certain HLA genotypes do not converge. This might point to different mechanics of non-progression. Until today, the VISCONTI cohort remains unprecedented because several participants even show decline of viral reservoirs years after treatment interruption. Other observations from treatment interruption trials as SPARTAC suggest that pre-treatment expression of PD-1, Tim-3 and Lag-3 on CD4 + T cells does strongly predict the time to the return of viremia (181). Because of its potentially broad applicability, biomarkers for functional CURE are a top priority in HIV-1 research today.

1.14 BARRIERS TO HIV CURE

Clearly, the barrier to a CURE is the viral reservoir. Its main characteristics are discussed extensively in chapter 2.9. In summary, the HIV-1 viral reservoir remains a hurdle mainly because of

1) Its **rapid establishmen**t within days post infection
2) Its **clonal expansion** (maintenance by proliferation of latently infected cells)
3) Its **tissue distribution**
4) Its **latent status**

How can these be potentially tackled?

Ad1) the WHO advises since the START study, early treatment initiation because it decreases the risk of transmission. However even when initiated during acute infection latency is not abolished (147). Overall, this emphasizes that early start of ART is desirable but does not suffice. In addition, a (very) early diagnosis is currently not realistic for most of the affected people outside of well-developed health care systems. However, the success of post exposure prophylaxis (PEP) exemplifies that there is a window of opportunity within 24 post exposure to even prevent infection (182). Still the rapidity of reservoir formation will not be easily targeted for most patients in the near future.

Ad2) there is currently no specific drug to target clonal expansion of latently infected CD4 + T cells. Interestingly this phenomenon is also observed in natural controllers so does not omit a status functional cure status (183).

Ad3) the tissue distribution of reservoirs poses several challenges. First, it comes with the fact that assessment of reservoirs in peripheral blood is not necessarily representative for the reservoirs in tissues (149). Secondly, the poor penetration of drugs into those tissues could be implicated into the low levels of resting viremia during ART (184). Whether this has an impact on shaping the reservoir during treatment remains controversial (115,185). In any case do barriers of peripheral reservoir

compartments get more and more into the focus of research. For example directing CD8 + T cells into B cell follicles, an immune privileged site of reservoir persistence in T follicular helper cells, has proven effective (159,186). The compound assessed in those studies is under current phase 1 clinical testing for safety in humans (NCT02191098). Similar strategies need to be developed for sanctuary sites of reservoirs such as the central nervous system (187).

Ad4) the main approaches to tackle viral latency to date are its permanent silencing on one hand or reactivation of the latent reservoirs for subsequent clearance. Both aspects will be discussed in the next section.

1.15 STRATEGIES AND INTERVENTIONS TO HIV CURE

Two elements play into ART free remission –the odds of viral reactivation from reservoirs and the extent of control in case of viral reactivation.

To lower the odds of viral reactivation shrinking the reservoir in size is the major objective to date (188). Different classes of latency reversal agents (LRA) have been investigated. Current limitations remain on the levels of their potency to reactivate reservoirs, their general toxicity and their ability to be used over multiple rounds of bursting (155). Clearly, a targeted depletion of reservoir containing cells would be probably the least toxic procedure. However, exclusive markers for viral reservoirs are not defined yet (189,190).

The level of control over reactivated virus can be increased by removal of susceptible target cells or strengthening of anti-viral immunity. The Berlin patient is the prominent example in which donor CD4 + T cells are insusceptible targets thus a barrier to viral rebound. Accordingly, gene therapy could come into action (191). Using bNAbs or therapeutic vaccination approaches for CD8 + T cells are only some options investigated to enhance anti-viral immune control.

Overall, a combinatorial approach might be most promising.

Two concepts of HIV-1 cure have gained broad acceptance in the field:

- The “shock and kill” approach combines the reactivation of viral reservoirs (shock) with a subsequently clearance of productively infected cells by immune responses or additional drugs. Most promising candidates to date are the toll-like-receptor (TLR) agonists. TLR7 agonist vesatolimod caused plasma viremia and reduced SIV DNA in blood and tissues leading to

sustained virological remission (158). These compounds are under clinical trial in HIV patients (NCT02588401 and NCT03060447).

- The “block and lock” strategy in contrast intends to silence the viral reservoir so it cannot be reactivated. The use of a Tat inhibitor for example lead to long term epigenetic silencing reducing viral RNA in tissues (192). Use of such molecules could be envisaged to reduce residual viral replication at first with long term silencing as a stepping-stone to stop ART at some point.

1.16 NATURAL CONTROL OF HIV-1 INFECTION

The by far biggest portion of the infected individuals do progress within several years to AIDS when not receiving ART. Only < 2 % of individuals spontaneously control HIV-1 infection halting progression to AIDS, the so-called long-term-non-progressors (LTNP), with stable CD4 T cell counts above 500. LTNP are further subdivided according to their pVL into elite controllers (EC) with viremia ≤ 20 copies per ml and viremic controllers (VC) with viral loads ≤ 2000 copies per ml of blood both for at least 24 months. Interestingly an observational study showed that once established, the maintenance of viral control was highly probable for 20 years and longer (193). The few controllers with emerging viral progression show signs of heightened inflammation and T-cell activation (194,195).

HIV controllers offer the unique opportunity to investigate the factors involved in long-term non-progression. This could ultimately develop new interventions leading to a functional CURE.

1.17 VIRAL DETERMINANTS

The residual viremia in EC and ART treated patients’ delivers crucial information regarding ongoing host-viral interactions. Whereas the pVL is comparable between EC and ART, the HIV-1 reservoir (here the cell associated total HIV-1 DNA) is significantly lower in controllers (196,197). In ART patients residual viremia might be linked to insufficient drug concentration in tissues (198). In natural controllers, the source of residual virus is not clearly determined. Longitudinal sequencing showed that the genetic evolution occurs primarily in plasma viruses and not in proviral clones so that ongoing replication does not lead to re-seeding and expansion of reservoirs (199).

Several studies described Nef defective or weaker replicative viral strains being overrepresented in HIV controllers (200,201). Importantly, natural long-term suppression was shown to be associated with inefficient reactivation of viruses from a limited number of infected cells (202). However more

recent literature retraces this as not the cause but rather the consequence of a more efficient anti-viral immune response indicating initial bias (194,203–205). Interestingly many plasma isolated viral clones from EC show evolution in HLA restricted epitopes arguing in favor of ongoing immune mediated pressure during viral control (206).

1.18 IMMUNE DETERMINANTS

Genome wide association studies have identified strong genetic associations in the MHC class I region (207,208). Especially the impact of the peptide-binding groove points towards an involvement in CD8 + T cells. Nevertheless do natural killer cell immunoglobulin-like receptors (KIRs) interact with MHC-I molecules as well. Indeed, two KIRs (KIR3DL1, KIR3DS1) have been associated with better viral control when in coexistence with the HLA-Bw4-801 allele (209). Also do cases of natural resistance to HIV-1 infection link with KIR2DL2/KIR2DL3 heterozygosity in the absence of HLA-C1 highlighting the impact of NK cell MHC-I interaction on the course of the infection (210).

On the level of humoral immunity the antibody dependent cellular cytotoxicity (ADCC) which depends on the non-neutralizing antibody qualities as well as the surface expression of Fcγ receptors is greater in controllers (211,212). Interestingly while the titers of neutralizing Abs are similar between patient categories the ADCC trends higher in controllers (213–215). In addition some of the most promising bNAbs have been derived from controllers (216). How these bNAbs develop in the absence of high viral loads is striking. Recent studies suggest an inflammatory signature linked to viral RNA supporting the need for constant antigenic exposure to drive affinity maturation(217).

Taken together, immune correlates of HIV control exist on different levels of immunity which is reflected by the heterogeneous population of controllers. Much evidence substantiates of the role of anti-viral CD8 + T cell in natural control of HIV-1. To this end, the following paragraphs will discuss CD8 + T cell immunity against HIV-1 in more detail.

1.19 HLA GENETICS AND THE T CELL REPERTOIRE

Once undergone negative and positive selection in the thymus, naïve T cells patrol the body screening their environment. Although the exact nature of a response is shaped by many factors, recognition of the peptide MHC class I (pMHCI) complex by the TCR is the indispensable step initiating CD8 + T cell immunity. Consequently HLA-I genotype is a major determinant for which peptides or epitopes are displayed on the cell surface hence potential recognition motifs for CD8 + T cells. In fact, strong

evidence for the involvement of CD8 + T cells during HIV-1 natural control derives from its association with certain HLA-I genotypes (B*27 and B*57) (208,218,219). Mechanistically these HLA molecules have a specific makeup in their peptide-binding groove leading to an extremely stable interaction with the respective epitopes (208). Also was it reported that the lower array of self-peptides presented by those genotypes subjects their T cell compartment to less negative selection resulting in a richer landscape of total TCR variants (i.e. the T cell repertoire) and also more cross reactivity (220). However most people with this genetic background still progress normally to AIDS (208). These striking clinical differences might in part relate to the exact TCR variants emerging. The TCR is a heterodimer created by somatic recombination early during lymphocyte and T cell development so that each naïve T cell expresses a unique variant, called clonotype. Of note, different clonotypes can recognize the same epitope. Accordingly TCR clonotypes of patients sharing the same HLA class I genetic background thus targeting the same epitope differ between progressors and natural controllers (221). Despite its huge potential diversity of the T cell repertoire, total numbers of unique clonotypes emerging to an antigen challenge, are limited. In fact, the precise TCR clonotypes result from self-peptide presentation during negative central selection in early life as illustrated by the study of Košmrlj et al (220). Finally, the ability to cross recognize escape variants crucially depends on the TCR clonotypes and so delineates viral control from progression (221,222).

1.20 IMMUNODOMINANCE, FUNCTIONAL AVIDITY AND EPITOPE SPECIFICITY

Despite the vast amount of potential peptides derived from the proteome of a pathogen, the effector T cell population is skewed towards only a few epitopes. This immunodominance hierarchy allows to rank epitopes based on their relative frequency within the pool of total pathogen specific effector T cells. Hence, some T cell epitopes are much more abundant than others. A plethora of factors has been found to play into the development of immunodominance (223–225). Yet the by far most decisive impact has the binding affinity of epitopes to the HLA-I complex (223). On functional levels only one study has reported that dominant clones are more mature, cytotoxic but with diminished proliferation and cytokine production than subdominant ones (226).

Conserved regions of the HIV-1 proteome are preferably targeted by subdominant epitopes, while dominant epitopes are more variable and hence prone to immune escape (227–229). The rapid viral escape to CTL response during acute infection of HIV-1 further suggests that the immunodominance hierarchy drives the mutational escape and plays into the cards of long term viral persistence (230,231). For example does superior recognition of certain subdominant epitopes associate with low

viral loads although individuals share the same HLA-B*1503 background (232). Important differences with regard to immunodominance and progression show that most effective clonotypes with regard to viral inhibition were immunodominant in controllers whereas absent or only subdominantly present in progressors (221). All together the current evidence suggests that therapeutic interventions should aim at inducing co-dominant responses against conserved epitopes to lower the odds of mutational escape.

The affinity of the TCR and its cognate peptide-HLA-I complex correlates with immunodominance (233). Logically researchers have asked the question if there is a link between the TCR-pMHCI affinity and the functional response of a T cell. This concept of functional avidity is experimentally defined as the peptide concentration that elicits a half-maximal functional response such as IFN-y secretion. There is contrasting data with regard to functional avidity as a correlate of viral control. Several groups report that functional avidity increases during chronic infection and doesn't differ between progressors and controllers (197,221,234–237). On the contrary, other studies find a relationship between high avidity T cells and viral control (238–240). Overall, functional avidity is to date no clear correlate of HIV-1 control.

Epitope specificity of T cells to different parts of the HIV-1 proteome were associated with varying degrees of disease progression. Specificity to peptides from the *gag* protein have been most frequently reported to correlate with lower viral loads (241–243). Interestingly, while the overall breadth of the anti HIV-1 CD8 + T cell response has no implications, the breadth of *gag* specific responses is associated with better functionality of CD8 + T cells and lower *in vivo* viral loads (243,244). These observations could be related to the relative conservation (hence difficulty for immune escape) of many Gag epitopes. In addition, the kinetics of *gag* antigen presentation in infected cells are much faster than most other viral proteins which have to undergo synthesis and processing before (245,246). Evolutionary conserved epitopes are important candidates for vaccination approaches because they portray a far lower likelihood of immune escape as mutations come with a high cost in viral fitness. Those epitopes have been identified foremost in *gag* and *pol* proteins of HIV-1. However in *gag* specific immune responses only during natural control (247–250). In conclusion, most evidence substantiates on the importance of gag specific CD8 + T cell responses during functional anti HIV-1 immunity. The dominance of those epitope specific CD8 + T cell responses will most likely protect from progression.

1.21 POLYFUNCTIONALITY, CYTOTOXICITY AND *IN VITRO* INHIBITION

The main effector mechanism of Cytotoxic T lymphocytes (CTL) is direct cytotoxicity accomplished by the formation and signal transduction through the immunological synapse with the target cell. Degranulation of perforin and granzymes act on the target cells by induction of cell necrosis, but also programmed cells death (apoptosis) (Figure 13).

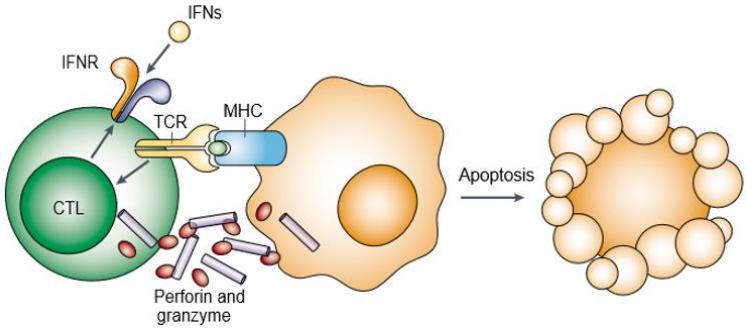


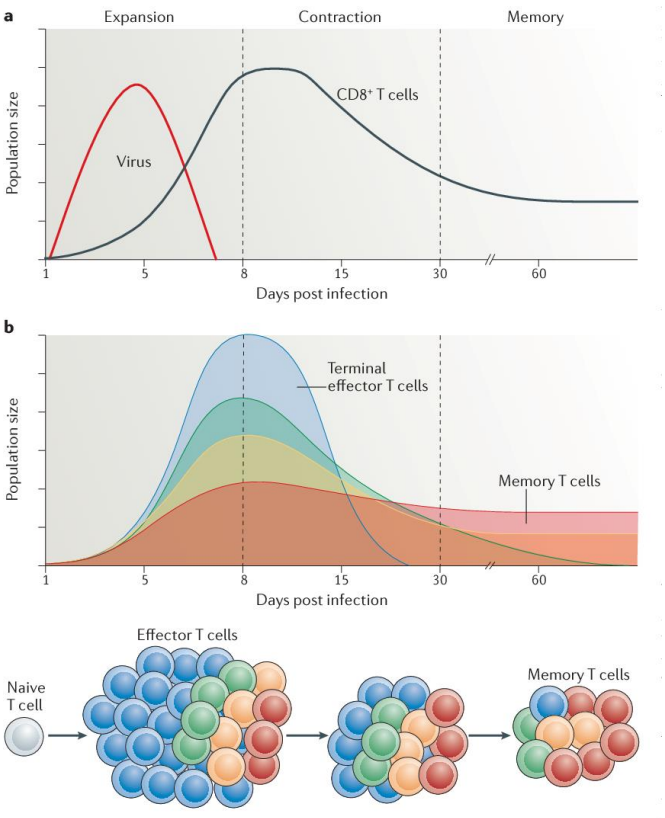
Figure 13. Schematic depiction of CTL mechanics in direct cytotoxicity (source: from creative-diagnostics.com) Cytotoxic T lymphocytes (CTL) or CD8 + T cells recognize epitopes presented in the context of MHC class I molecules. The functioning of those CTL is orchestrated by cytokines such as interferons (IFNs). In an activated state, the CTL forms an immunological synapse secreting perforin and granzyme, which in turn induce apoptotic cell death in target cells.

Alongside with their direct cytotoxic functioning CD8 + T cells can release non lytic factors such as cytokines which exert effects on the dynamics of the immune response (such as IL-2) or impede the viral life cycle directly (such as MIP1-beta) (251,252). Co-expression of multiple cytokines in conjunction with degranulation, termed polyfunctionality, is significantly increased in EC and was observed by multiple, independent studies over the years (239,253–255). Thus, the quality of CD8 + T cells delineates natural control from chronic progression. Previous studies also suggested that the magnitude and breadth of HIV-1 specific CD8 + T cells does not differ between progressors and LTNP (256,257). However as soon as treatment is started in progressors and viral loads sink below detectability the number of HIV-1 specific CD8 + T cells decreases overtime (258). Why in contrast natural controllers maintain relatively high numbers of CD8 + T cells specific to HIV-1 although similar pVL as treated patients is poorly defined. Recent studies highlight the importance of lymph node resident follicular CD4 + T helper cells in the ongoing viral replication probably linked to inhibition of cytotoxic CD8 + T cell functions (259). Studies in SIV infection suggest a functional role of B-cell follicle homing CD8 + T cells (CXRCR5+) although their contribution to control SIV infection might be comparably minor (260). To what degree CXCR5+ CD8+ T cells are present in EC and which functional phenotype they have in HIV-1 infection remains to be investigated.

Similarly to polyfunctionality, cytotoxicity, measured mainly by the expression of Granzyme B and Perforin with flow cytometry, is increased in CD8 + T cells of controllers as well as their proliferation (261–264). The capacity of anti-viral CD8 + T cell effectors to inhibit virus directly can be measured *in*

vitro by determination of viral inhibitory activity (VIA), typically determined using viral suppression assays. Common suppression assays use an autologous co-culture system of superinfected CD4 + T cells, the target cells, with CD8 + T cells, the effector cells. Longitudinal measurements of viral outgrowth monitors the VIA for each patient (265). Collectively all studies observe an increased capacity in EC compared to treated or untreated progressors (248,262,266–269). In addition, several clinical parameters were correlated to VIA. So does for example a decreased VIA predict faster CD4 T cell decline in progressors (160). Long term viral control in contrast correlates positively with VIA (161). Noteworthy the frequency of *gag* specific CD8 + T cell responses also correlate with increased VIA (248). Again does this body of evidence support the hypothesis of augmented anti-viral CD8 + T cell functioning and natural viral control. Although extensively studied the precise functional phenotype of CD8 + T cell subsets responsible for viral inhibition is still cumbersome.

1.22 T CELL EFFECTOR AND MEMORY FORMATION



The T cell response to acute viral infections runs through three distinct phases (Figure 15). First, a profound expansion takes place during which the antigen specific naïve CD8 + T cells massively proliferate and evolve into effector cells, acquiring functions such as the production of cytotoxic molecules. Next, after the antigen clearance (Figure 14 a, day 8), the contraction phase follows, during which most effector cells die by apoptosis. Third, the formation of immunological memory from a small portion of effector cells that persist as long living T cell memory, takes place.

Figure 14: The three different phases of a specific T cell response (adapted from Kaech et al. Nature Immunology Review). Timeline of the magnitude of the viral titer in relationship to T cell response phases (a). Prevalence of different effector cell subsets over the course of the T cell response. Note that the color code indicates that memory cell potential is not equivalently inherent in all subsets of effector cells (b).

It is this cellular memory, which is a defining characteristic of adaptive immunity allowing a rapid re-emergence of effector T cells in case of re-exposure to the pathogen. Chronic and persistent antigen presence during untreated HIV-1 infection by contrast prevents a proper contraction of effector T cells. Consequently, HIV specific CD8 + memory T cells show altered differentiation patterns and are mostly in pre-terminally differentiated memory subsets (272,273). Also do $CD28^{-}$ $CD27^{+}$ T cells dominate HIV specific memory CD8 + T cells, a phenotype which correlates with lower proliferative and cytotoxic potential (274).

In line with these findings is the constant elevation of T cell activation markers during chronic disease (275). Molecules such as CD38 and HLA-DR on CD4 + and CD8+ T cells can indicate chronic activation during high, uncontrolled viremia and have been associated with poor prognosis in untreated progressors. However, the same markers have been proposed as identifiers of efficient CD8+ T cell mediated viral suppression in natural controllers (269,276,277). These findings suggest that phenotypic markers have to be carefully contextualized and interpreted in conjunction with functional assays.

1.23 T CELL EXHAUSTION AND SENESCENCE

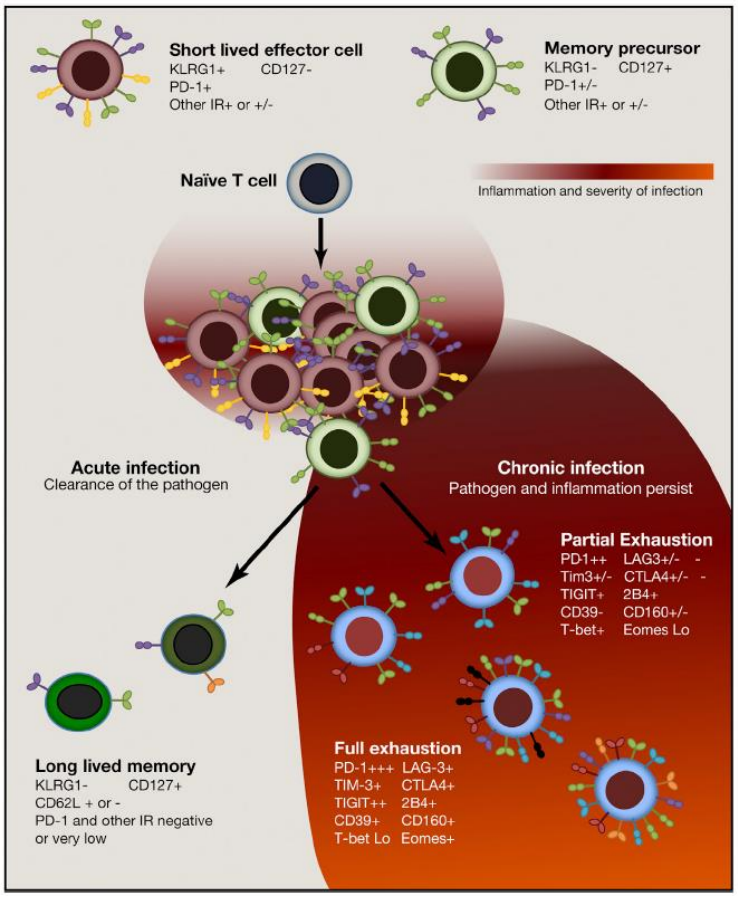


Figure 15. The differentiation pathway and development of exhausted T cells (adapted from (290)). Expansion of virus specific CD8 + T cells and development into effector cells which contain memory precursors and short-lived effector cells. If the infection becomes chronic and there is no clearance the memory precursors cannot properly differentiate and instead become persistently triggered by the present antigen leading to exhaustion. The grade of exhaustion correlates with co-expression of inhibitory receptors and certain transcription factors.

Exhausted T cells arise as a distinct lineage of effector T cell during chronic antigen persistence. They are among other aspects characterized by loss of effector functions with elevated and sustained co-expression of inhibitory receptors (IR) (278). A continuously growing number of IRs is characterized of which PD-1 and CTLA-4 are the most studied (Figure 15). The observation of functionally impaired late stage effector T cell subsets goes back to studies in LCMV infected mice in which also its potential reversal was successfully demonstrated (279,280). Since then exhausted T cell subsets were identified and studied in various human chronic infections but also in cancers. Loss of effector functions occurs in a hierarchical manner during which IL2 production is affected first followed by TNF-α (252,281). At this stage, also the cytotoxicity can become compromised. Lastly the terminally exhausted T cells lose the ability to produce and secrete IFN-γ (282–285). It is important to keep in mind that although impaired in function exhausted T cells are not inert (286). In general, severity of exhaustion correlates with the pathogen burden. PD-1 expression for example on HIV specific CD8 + T cells is positively correlated with pVL and negatively with CD4 T cell counts. The same relationship is reported for its expression on the general CD4 + T cells (287). As could be expected, levels of IR expression on T cells decline during ART (288). But their expression stays elevated compared to uninfected controls and essential transcription factors, T-bet and EOMES, remain altered even during long term suppressive therapy (289).

Intriguingly, however, functional avidity on CD8 + T cells correlates strongly with PD1+, CD160+ and 2B4+ coexpression during untreated infection in natural controllers as well as in progressors (234). The same co expression profile was correlated to higher VIA (291). This goes to show expression of so-called IR is not always indicative of exhaustion, but needs to be contextualized. Many aspects of exhaustion remain to be understood.

As discussed in the previous chapter memory formation occurs upon the clearance of the antigen involving IL-7 and IL-15 signaling for long term memory T cell maintenance (292). In contrast exhausted T cells show loss of IL-7 and IL-15 sensing and are instead maintained by the persisting antigen. Consequently the antigen dependent altered memory homeostasis is likely also a driver of the observed loss of CTL responses upon viral escape (293). The hidden potential of exhausted T cells is impressively demonstrated by the clinical success in cancer immunotherapies. Due to the tremendous impact of IR blocking antibodies against PD-1 and CTLA-4 in cancer therapy, their initial discovery was rewarded with the Noble Prize in Physiology and Medicine of 2018.

The diminished effectiveness of the aging immune system is labelled as immunosenescence. Its cardinal features are the impaired ability to respond to new antigens, unsustained memory responses, higher incidence of autoimmunity and a lingering low grade of inflammation (294). The limited success of vaccination in the elderly is one of possible examples and probably linked to the loss of the naïve T cell compartment (295). As already discussed in the section on immunopathogenesis the strong inflammatory stimuli by HIV-1 infection hits the full system. Therefore some aspects of an aged immune system such as higher levels of circulating cytokines are prematurely found in HIV-1 infected individuals even under therapy (53,296). In the subsequent paragraph, we focus rather on the aspect of T cell senescence which refers to absent or poor replicative capacity in the T cell memory compartment.

As every cell can divide only a finite amount of times due to telomere shortening, it is hypothesized that IR signaling during exhaustion serves as a physiological feedback to block unceasing T cell proliferation. By that, replicative senescence is precluded, which can lead to the loss of T cell clones from the repertoire (297). In fact it is important to emphasize that T cell exhaustion and T cell senescence are two different paths of differentiation (298). This is reflected in the mostly non-overlapping expression of surface markers such as CD57 for senescence and PD-1 for exhaustion. In addition, do senescent cells show a pro-inflammatory profile with cytokine expression which is clearly no feature of exhaustion (299). The identification of senescent cells is possible by looking at different

phenotypic markers. An accumulation of CD28 and CD27 double negative CD8 + T cells is observed in aging individuals and repetitive antigen exposure. Senescent subsets show lowered telomerase activity, short telomers and proliferative arrest (300). Another important phenotypic marker is CD57. Its expression on CD8 + T cells been seen in cytomegalovirus (CMV) infection and is associated with immune aging (301). During HIV-1 infection CD57 identifies late differentiated subset with increased VIA but lack of proliferation (291).

Overall, the anti-viral CD8 + T cell response associates with long term control of HIV-1 infection in absence of ART. Although a plethora of aspects has been studied in natural controllers, the detailed identification of cellular subsets linked to strong VIA is still lacking.

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2 Scope and objectives of the thesis

Globally nearly 38 million people live with HIV-1 infection today (UNAIDS, 2019). Although viral replication is effectively suppressed by drug therapy, the infection remains incurable to date. The main barrier to cure HIV-1 is its persistence in viral reservoirs (chapter 2.9). Therefore, rapid rebound of viremia upon treatment cessation is commonly observed in most patients. Current evidence highlights that a "functional cure", control of HIV-1 infection in the absence of therapy, can be possible (chapter 1.10). Of note, reports on post treatment control or PTC define a reduced size in viral reservoirs as a common attribute but clear correlates of viral control upon treatment interruption are still lacking (181,182).

The heterogeneity of viral reservoirs and distribution over various tissues of the human body is well documented (chapter 1.9). In turn characterization and measurement of reservoirs has been challenging as latently infected cells are extremely rare in peripheral blood which is not representative of tissues. To this end, cellular biomarkers of HIV reservoirs would allow not only a better understanding of various aspects of viral latency but also its specific targeting. In this context the third chapter presents a study of HIV-1 infected humanized mice with the objective to:

- Develop and validate a humanized mice model of HIV-1 infection and viral latency by oral ART
- Monitor differences in early and late treatment regimens with regard to immune restoration, reservoir size and viral rebound upon treatment cessation
- Specifically investigate the role of CD32 concerning its expression during latency in various tissues and its controversially debated role as a biomarker of the HIV-1 reservoir.

The "shock and kill" approach of the HIV cure field postulates a purging of viral reservoirs by latency reversal followed by host immune mediated clearance (chapter 1.10). Functional HIV-1 specific CD8 + T responses are characterized as a powerful element of anti-viral immunity (chapter 1.12). To that end, chapter four reports on a study which intended to:

- Evaluate the potential of HIV-1 peptide stimulation at inducing CD8 + T cell mediated inhibition of viral replication *in vitro* in a cohort of virally suppressed progressors on ART
- Identify correlates of enhanced viral inhibition with potential application in therapeutic vaccination strategies.

Finally, a role model of HIV-1 containment are rare individuals controlling HIV-1 infection in the absence of therapy (chapter 1.11). Although enhanced capacities of their anti-viral CD8 + T cell compartment has been repeatedly reported (chapter 1.12), the detailed phenotype of CD8 + T cells linked to increased viral suppression is not defined. Chapter five presents the results on a study which aimed at

- Comparing the effect of HIV-1 peptide stimulation on viral inhibition of replication *in vitro* across different clinical phenotypes of spontaneous HIV controllers
- Identifying functional CD8 + T cell subset which are potentially implicated in inhibition of viral replication and during *in vivo* control of HIV-1

Taken together, the central hypothesis of the presented work is that biomarkers of the viral reservoir in combination with correlates of CD8 + T cell mediated viral control are important stepping stones towards a functional cure of HIV-1 infection.

The final chapter six discusses the presented data in perspective of HIV cure but also the general global challenges in HIV/AIDS.

3 $CD32^{+}CD4^{+}$ MEMORY T CELLS ARE ENRICHED FOR TOTAL HIV-1 DNA BUT ARE A NEGLIGIBLE TRANSLATION-COMPETENT VIRAL RESERVOIR IN HUMANIZED MICE

Author's contribution: The following chapter was a common work with the co-first author of the article, Virginie Fievez, who had implemented the humanized mouse treatment model in the lab. Furthermore was the HIV CURE research center in Ghent involved in the reservoir measurements (total HIV-1 DNA and RNA) by droplet digital PCR.

$CD32^{+}CD4^{+}$ memory T cells are enriched for total HIV-1 DNA but are a negligible translation-competent viral reservoir in humanized mice

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Manuscript to be submitted to the journal of virology after approval by co-authors

3.1 ABSTRACT

The elimination of the latent HIV reservoir remains the main hurdle to HIV cure. Since CD32 was proposed as a marker of the replication-competent HIV-1 reservoir, a number of conflicting results were raised. In this study, we deciphered the expression of CD32 in tissues from HIV-1 infected and virally suppressed humanized mice. Humanized mice treated with early ART (one week after HIV infection) or late ART (8 weeks after HIV infection) replicated important features of HIV-1 pathogenesis and latency. Using a strict flow cytometry gating strategy for $CD4^+$ T cells, we showed that CD32 was consistently expressed in a small fraction of the memory $CD4^+$ T cells subsets from different tissues and the proportion of the CD32+ fraction did not differ significantly between untreated and mice treated early or late. Interestingly, $CD32^+$ memory $CD4^+$ T cells were enriched in cell-associated (CA) HIV-1 DNA but not in CA HIV-1 RNA as compared to the $CD32^-$ fraction. CD32 expression was not associated with the CA HIV-1 DNA content but correlated with the expression of activation markers in memory $CD4^+$ T cells. Using multidimensional reduction analysis, several distinct clusters of the memory $CD4^+$ $CD32^+$ T subpopulations co-expressing either HLA-DR, CD38, TIGIT or PD-1 were identified. Importantly most of the translation-competent viral reservoir was found in the $CD32^-CD4^+$ T cells fraction although the small number of $CD32^+$ $CD4^+$ T cells displayed higher frequency than $CD32^-$ $CD4^+$ T cells. By providing insights into the early establishment of the viral reservoir in tissues, our findings support that CD32 is not a marker of the total HIV-1 DNA content in $CD4^+$ T memory T cells but represents highly activated memory CD4+ T cell subsets that contain only a small proportion of the productive reservoir.

3.2 INTRODUCTION

The advent of antiretroviral therapy (ART) has dramatically improved the clinical care of HIV-1 infection. In 2015 the START trial demonstrated that immediate initiation of ART was superior in reducing serious AIDS and non-AIDS related events (1). Since then treatment guidelines advice to start therapy as early as possible (2). Although beneficial in reduction of morbidities, early treatment is not curative due to the establishment of long-lived viral reservoirs (3–6). By definition viral reservoirs are cell compartments or anatomical sites containing replication-competent virus (7). Its latent fraction is transcriptionally inactive therefore refractory to ART.

HIV-1 reservoirs are rapidly established upon primary infection. In Simian Immunodeficiency Virus (SIV) reservoirs are detectable as early as three days post-infection in tissues even before detectable viremia in circulation (8). Likewise, initiation of ART during the acute phase of infection in humans doesn't impede reservoir formation and viral rebound after treatment interruption (5,6). In addition the latent cellular reservoir is extremely stable with an estimated half-life of 43.9 months so that prolonged ART cannot eliminate HIV-1 in a patient's lifetime (9–12). Approximately 1 in 10^5-10^8 of total $CD4^+$ T cells is latently infected, of which about only 1% is replication competent (13–15). This scarcity leads to many technical challenges faced to precisely determine and study HIV-1 reservoirs. Initially localized in resting central memory (CM) $CD4^+$ T cells (16–18) today the heterogeneous dissemination of reservoirs over all $CD4^+$ T cell compartments is well recognized (19–21). Furthermore the tissue distribution of reservoirs is diverse and poorly reflected by recirculating $CD4^+$ T cells in peripheral blood (22,23). Taken together biomarkers of latently infected cells are urgently needed to allow a more precise characterization of reservoirs and develop strategies for their targeted depletion.

Various surface markers (CXCR3, CCR6, CD2, PD-1, LAG-3, TIGIT) were proposed to identify latently infected cells but studies have not converged (19,24–28). Recently CD32, a low affinity Fc-γ-RII receptor, was proposed as a putative marker identifying a subset with high enrichment in total HIV-1 DNA and replication competent provirus (29). Since then studies provided controversial data (30–34). $CD32^+$ $CD4^+$ T cells were shown to harbour a higher activation profile and to correlate with general immune activation (26). Overall data from several patients cohort did not substantiate on the relevance of CD32 expression in latent HIV reservoir probably due to the heterogeneity of ART initiation's timing, ART duration, and levels of immune activation. Interestingly recent emphasis was put on the different purity of $CD32^+CD4^+$ T cells across various studies as a possible explanation for

divergent findings (30,35). Furthermore, only few studies have scrutinized the role of CD32 in peripheral tissues.

Molecular mechanisms of HIV-1 latency are mainly studied in cell lines but have very limited meaning to clinical settings (36–38). Instead, humanized mice models have earned their place in HIV-1 research as versatile tools to study HIV-1 pathogenesis *in vivo* (39). To date ART treatment has reproduced the clinical features of HIV latency in these small animals (40,41). A critical advantage is the easy access to all anatomical sites and precise control over timing and duration of HIV infection or ART for a complete understanding of HIV-1 latency.

To this end, we have developed an HIV latency model in NSG humanized mice recapitulating the early establishment of the viral reservoir. The present study scrutinized the role of CD32 expression in viral reservoir formation and its relationship to immune activation. We show here that CD32 expression was low in both $CD4^+$ and $CD8^+$ T cells from different tissues in humanized mice treated relatively early with ART (one week after HIV infection) or late (8 weeks after HIV infection). $CD32^+$ memory $CD4^+$T cells were enriched in cell-associated HIV DNA but not in cell-associated HIV RNA. $CD32^+$ $CD4^+$ memory subsets associated with their activation/exhaustion status under ART. Importantly most of the productive HIV reservoir was comes from the $CD32^-$ memory $CD4^+$T cells fraction. Taken together, our data support that CD32 expression describes an activated $CD4^+$ T cell memory subset enriched with proviral DNA independently of treatment initiation. Ultimately, we showed that CD32 does not clearly label the replication competent HIV-1 reservoir that needs to be eliminated towards HIV cure.

3.3 MATERIAL AND METHODS

NSG mice immune reconstitution, HIV-1 infection and treatment with ART

NSG (NOD/LtSz-scid/IL2Rγnull) mice were purchased from Jackson Laboratory, USA. All animal experiments were performed in accordance with the Animal Welfare Structure of the Luxemburg Institute of Health (LIH) (protocol number: LRTV 1402) and complied with the national legislation and guidelines for animal experimentation. Juvenile (3-4 weeks of age) NSG mice were engrafted as previously published (42). Briefly, mice were conditioned by two intraperitoneal Busulfan injections (20 mg/kg, Busilvex®) 24 and 12 hours before transplantation. CD34+ hematopoietic stem cells were isolated from human cord blood, provided by the Blood Bank of the University Hospital (Liège, Belgium), by magnetic activated cell sorting (MACS), using the CD34+ progenitor cell isolation kit (Stem Cell Technologies, Vancouver, Canada). Subsequently $2x10^5$ freshly isolated CD34+ cells were transplanted into each NSG mouse by intravenous injection. Mice were bled at 12 and 24 weeks post-transplantation to determine human immune cell reconstitution by flow cytometry.

Animals with ≥ 20% of circulating human $CD45^+$ cells at 24 weeks post-transplantation were infected by two intraperitoneal injections of a HIV-1 laboratory adapted strain (JRCSF, 10.000 TCID50) within 24 hours. Infection was monitored every 2 weeks on blood samples collected by submandibular bleeding for $CD4^+$ T cell counting using flow cytometry analysis and for viral load measurement (see below). ART treatment was initiated 1 week (early treated) or 8 weeks (late treated) post infection and continued for a total of 8 weeks for both groups using a single tablet regimen of dolutegravir/abacavir/lamivudine (Triumeq®, ViiV Healthcare) provided in the drinking water. The tablets were crushed and dissolved in Sucralose MediDrop® (Clear H_2O, Portland, USA) at the therapeutic concentration of 3.40 mg/ml. Drinking solution was refreshed twice per week and volumes were monitored to follow up consumption. At the end of the protocol, mice were anesthetized using a mixture of ketamine and xylazine, blood samples were collected via cardiac puncture and tissues were harvested for downstream analysis.

Experimental design

The study was structured into three arms (Figure 1). A first group of mice infected with HIV-1 receiving treatment from 1 week post infection on (early treated, n= 58). The second group was set on therapy at week 8 post-infection (late treated, n=42) and a third group of HIV-infected mice never received antiretroviral drugs (untreated, n=20). After 8 weeks of ART treatment, animals were sacrificed (n= 43 and 34 in early and late treated groups). Untreated animals were sacrificed at the matched time points

with the early and late treated mice respectively (n=10 for each group). Some of treated animals were kept while ART was discontinued to measure time to viral detectability post ART (viral rebound), (early treated n= 15; late treated n=8). Monitoring of viral load upon HIV-1 infection and under ART treatment was performed using absolute ddPCR measurement every two weeks.

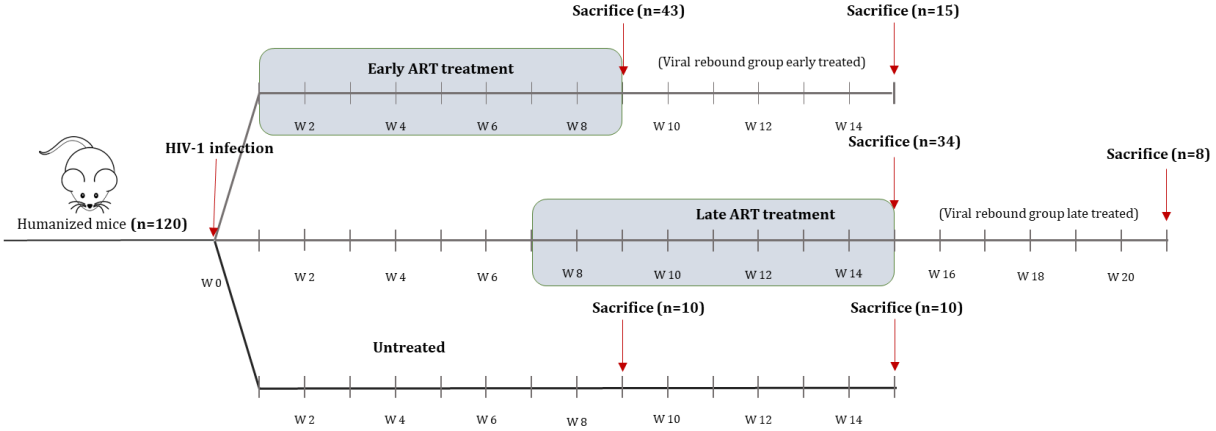


Figure 1. Design of the study: 120 humanized NSG mice were infected with HIV-1. At week one post infection ART regimen was initiated (n=58) in the early treated group and sustained for 2 months. Late treatment was started at 8 weeks post infection (n=42) and continued for 2 months as well. In each group, a number of animals was sacrificed at the end of treatment (early treated (n=43) week 9 and late treated (n=34) week 16). The untreated group (n=20) never received ART and was euthanized correspondingly to sacrifices of both treatment groups (week 9 for early treated and week 16 for late treated). Viral rebound after treatment interruption was monitored for early and late treated animals for a duration of 6 weeks or longer if animals were not detectable yet.

Organ collection, processing and isolation of cells

Organs were collected at necropsy, washed in cold RPMI and processed immediately for cell isolation. Lymph nodes were dissected from the axillary and intestinal locations. Spleens and lymph nodes were gently disrupted in cold PBS using the plunger of a syringe. Bone marrow (BM) were obtained from tibias and femurs. Both ends of bones were opened and BM flushed out by short centrifugation at 600g during 30 seconds at room temperature. BM cells and splenocytes suspensions were passed through 70 µm nylon cell strainers (BD Biosciences, Franklin-Lakes, New Jersey, USA) and centrifuged 10 min at 400g and 4°C. Splenocytes were resuspended in fresh ACK (Ammonium-Chloride-Potassium) solution (in house made) for 5 minutes to lyse red blood cell followed by two washes with RPMI 1640 (GIBCO Life technologies, Thermo Fisher Scientific).

Quantification of HIV-1 plasma viral load

Plasma viral load were measured by droplet digital PCR (ddPCR) using the Bio-Rad C-1000 Thermo cycler (Bio-Rad laboratories, Hercules, CA, USA). Primers and probes were designed as reported previously (43). Input volumes of 100 µl were achieved for RNA extraction using the MagNa pure kit (MagNa Pure Compact nucleic acid isolation kit, Roche, Basel, Switzerland) according to the manufacturer's protocol by diluting 30 µl of mice plasma in 70 µl of PBS. The limit of detection is 235 copies/ml of blood (33). Data were analyzed with the QuantaSoft software (Bio-Rad laboratories, Hercules, CA, USA).

Quantification of total HIV-1 DNA and CA-RNA from human CD45$^+$ cells

Human CD45$^+$ cells from organs were purified by positive selection using magnetic beads (CD45 MicroBeads, Miltenyi Biotec, Bergisch-Gladbach, Germany) following the instructions of the manufacturer and pelleted. Total DNA was extracted from spleen, lymph nodes, lungs and bone marrow cells by manual extraction using the NucleoSpin® Tissue kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. Aliquots of eluted samples were frozen at −80 °C until total HIV-1 DNA quantification by ddPCR as previously reported (97). RNA was isolated from human CD45$^+$ cells using the innuPrep RNA Mini Kit 2.0 (Westburg, Berlin, Germany), then reverse transcribed into cDNA using qScript cDNA SuperMix (Quanta bio, Beverly, Massachusetts, USA) in a total volume of 20µl according to manufacturer's protocol. Cell-associated HIV-1 RNA (CA-RNA) was measured by ddPCR (Bio-Rad, Hercules, California) in triplicates as previously described (44). Three reference genes (B2M, ACTB, and GAPDH) per cDNA samples were assessed by qPCR to normalize for RNA input. Copies of CA-RNA were divided by the geometric mean of the reference gene related to the theoretical number of cells (expressed in copies per million cells).

Flow cytometry and cell sorting

Detailed phenotyping of human B and T cells was performed using 2x10^6 single cells from each organ. Cells were washed with PBS supplemented with 1%FBS (Gibco™, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and stained for 30 min at 4°C with fluorochrome-labeled monoclonal antibodies. Antibodies of human reactivity if not indicated differently: CD4-BUV395 (SK3), CD3-BUV496 (UCHT1), CD8-BUV805 (SK1), CD279-BV421 (EH12.2H7), CD28-BV605(CD28.2), CD27-BV650(M-T271), CD197-PECF-594 (150305), murineCD45RO-PECy5 (30-F11) from BD Biosciences and HLA-DR-BV510 (L243), CD127-BV711(HIL-7R-M21), CD32-PE (FUN-2), TIGIT-APC (A151536) from Biolegend (San Diego, CA, USA) and LIVE/DEAD fixable Near-IR cell dye (Thermo Fisher Scientific). Cells were fixed for 1h at 4°C

using 1X BD Lysing solution (BD Biosciences, Cat n°: 349202) before acquisition. Samples were acquired on a FACS Fortessa SORP 5 laser instrument (BD Biosciences) and analyzed with the Kaluza (version 2.1, Beckman Coulter, Brea, California, USA) and Flow Jo (version 10, BD Biosciences) softwares. Four-way cell sorting was done by staining single cells derived from organs with CD3-BV510 (UCHT-1), CD4-PECy7 (RPA-T4), CD8-BV711 (RPA-T8), CD197-APC (3D12), CD45RA-FITC (HI100), murineCD45RO-PECy5 (30-F11) (BD Biosciences) and CD32-PE (FUN-2) (Biolegend) and LIVE/DEAD fixable Near-IR cell dye (Thermo Fisher Scientific). CD4+CD45RA- memory T cells were sorted based on their expression of CD32 into positive and negative subsets using BD Influx instrument (BD Biosciences). The detailed gating strategies for phenotyping and cell sorting on CD4+ T cells for strict exclusion of CD19, CD14 and T-B cell doublets or conjugates are given in supplementary Figure 3.

FlowSom clustering

To investigate the relationship between CD32 expression and the activation status of $CD4^+$ and $CD8^+$ T cells, virally suppressed mice (early treated (n= 12) or late treated (n= 10) were phenotyped at 4 weeks and 8 weeks of treatment. Bone marrow and spleen were harvested and flow cytometry was performed as described above. FCS files were compensated and cleaned in Flow Jo (version 10, BD Biosciences), exported and uploaded into the Cytobank environment (Beckman Coulter). After proper scaling FlowSom was run on $CD3^+CD14^-CD19^-$ T cells using all markers. Clusters were represented as median expression heatmap by hierarchical, agglomerative organization. Statistical analyses was done using Kruskal Wallis

Quantification of translation-competent viral reservoirs

The HIV-Flow assays was used as previously described (46) to quantify the amount of the translation-competent HIV reservoir in human $CD4^+CD32^+$ versus $CD4^+CD32^-$ T cells. Briefly, cells were harvested from spleen and bone marrow of HIV-infected ART suppressed mice (late protocol, as described above) and cultured in RPMI + 10% Fetal Bovine Serum supplemented with antiretroviral drugs (200nM raltegravir, 200nM lamivudine) . A protein transport inhibitor (Golgi Plug, BD Biosciences) was added according to manufacturer's instruction 1 hour before stimulation and maintained during stimulation with PMA/Ionomycin (both Sigma, Belgium @162nM/ml and 1µg/mL respectively) for 24 hours. Cells were stained for surface markers and the intracellular p24 protein (anti-p24 PE, clone: KC57, Beckmann Coulter and anti-p24 APC, clone: 28B7, MediMabs, Canada) using the BD Cytofix/Cytoperm staining kit (BD Biosciences).

Extracellular expression of CD32 combined with activation/exhaustion markers and the intracellular staining of p24 was performed as described above.

Statistical analysis

Statistical analysis was performed using GRAPHPAD PRISM software. Groups were compared using nonparametric Mann-Whitney *U* test or Kruskal Wallis with Dunn correction for multiple comparison. A p-values $< 0,05$ was considered to be significant (Number of stars indicating p values: * ≤ 0.05, **≤ 0.005, *** ≤ 0.0005, **** ≤ 0.00005. Abbreviation ns stands for non-significant result). Two-tailed Spearman correlation coefficient was calculated for several comparisons (r and p values indicated on respective graphs). Mantel-Cox rank test was applied for Kaplan Meyer curve comparison.

3.4 RESULTS

In this study, early treatment was started 1 week and late treatment 8 weeks after HIV-1 infection. In each group, treatment was maintained for 8 weeks (see figure 1). As expected, a sharp increase of plasma HIV-1 RNA in the range of 10^4-10^5 copies per ml was detected from one week after infection on in all animals (Figure 2, A). Plasma viremia was strongly reduced within 2 weeks of treatment (figure two, A). Interestingly early treatment led to a significantly more rapid time to viral suppression i.e. undetectability of the pVL assay ($p<0.0001$ see Figure 2, C). Conversely, the kinetics of viral rebound after treatment interruption were similar in both groups (Figure 2, D) although some early treated mice (i.e. 4/15) harboured transient viremia blips and returned to undetectable viral load 6 weeks after treatment cessation (supplementary figure 1). These observations indicate the establishment of replication competent viral reservoirs within one week of infection that persist during prolonged ART.

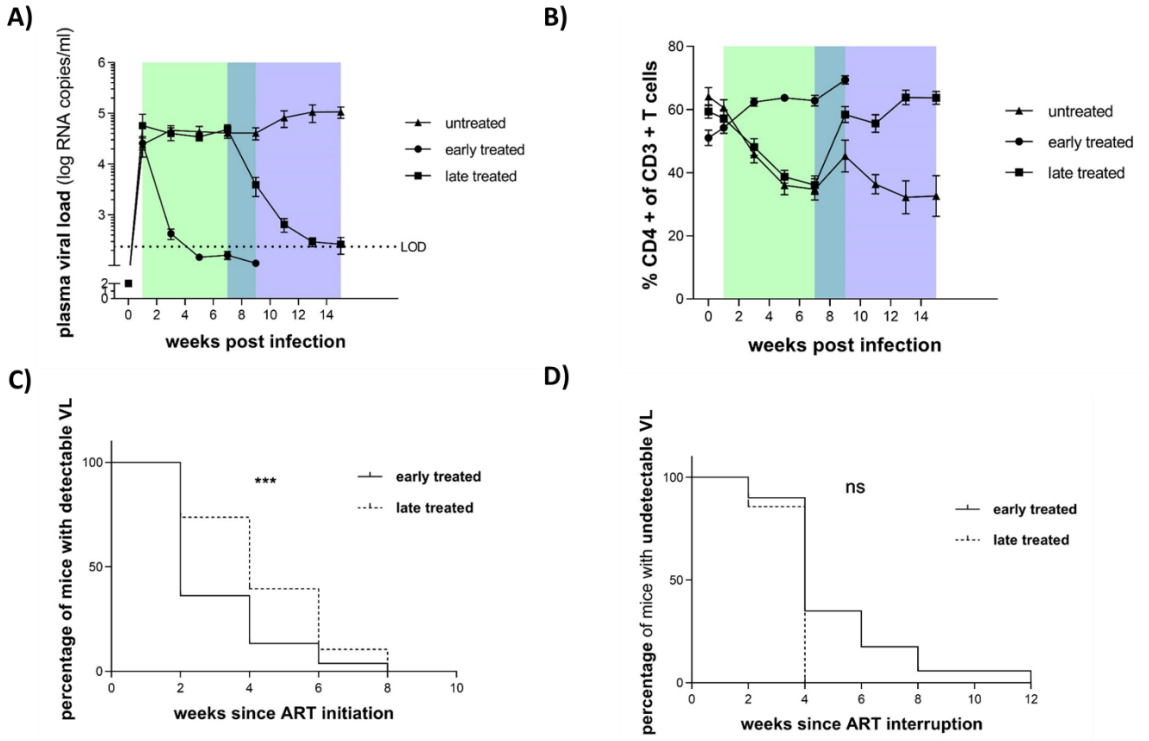


Figure 2. Early treatment significantly decreased time to viral suppression whereas CD4$^+$ T cell restoration and viral rebound were similar between both groups

Plasma viral load of early treated (n=43), late treated (n=34) and untreated (n=20) (A) and percentage of CD4$^+$ T cells (B) were determined every two weeks in peripheral blood. Time to viral suppression (median 2 and 4 weeks respectively) ($p< 0.0001$) (C) and viral rebound ($p = 0.107$) (D) for the early and late group. Statistical testing executed using Mantel COX rank test. (* $p< 0.05$; *** $p< 0.01$; *** $p < 0.001$; **** $p < 0.0001$)

The immunological hallmark of HIV-1 infection is the depletion of CD4$^+$ T cells. We observed a rapid decline in circulating CD4$^+$ T cells upon infection. At week three post-infection, untreated humanized mice clearly lost around one third of their human CD4$^+$ T cells whereas early treatment prevented CD4$^+$

T cell loss. After late treatment onset, a gradual recovery of $CD4^+$ T cells was observed starting from two weeks on. At the end-point (week 16), peripheral blood $CD4^+$ T cell counts were restored to similar levels as in early treated mice (figure 2B). These data suggest a maintained restorative capacity of the human T cell compartment in this model.

Total HIV-1 DNA is significantly reduced by ART treatment and early initiation

The stable persistence of provirus during therapy is a defining factor of HIV-1 latency. Therefore, we analysed four different organ sites for the presence of total HIV-1 DNA by ddPCR at the end of ART or respective time points for the non-treated animals (see figure 1). ART led to a significant reduction of total cell associated DNA in various organs compared to the untreated control group (Figure 3). Furthermore, early initiation of ART reduced significantly total HIV-1 DNA as compared to late initiation in bone marrow (1.82 and 3.85 median log_{10} copies/million cells, respectively) and lymph nodes (2.45 and 3.7 median log_{10} copies/million cells respectively). Trends were similar for spleen but not significant. These data underline the recapitulation of reservoir establishment and persistence during ART treatment in humanized mice.

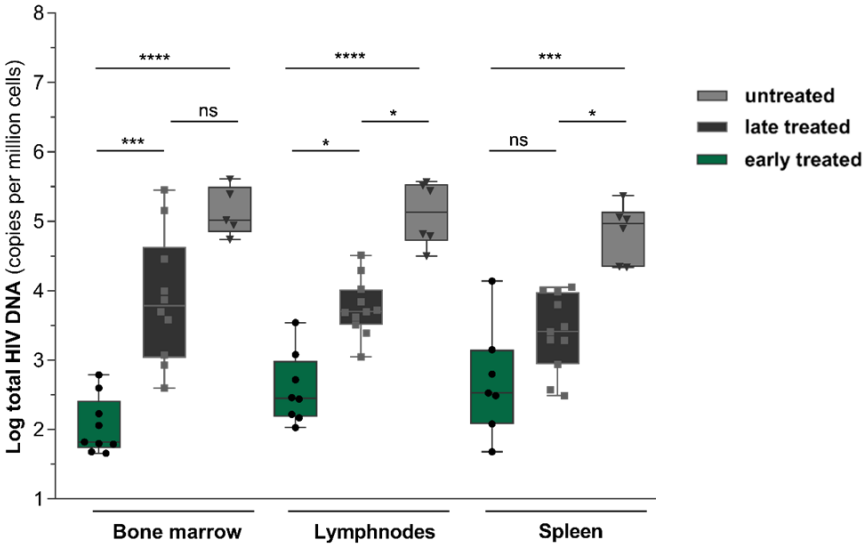


Figure 3. ART significantly decreased total HIV-1 DNA in peripheral organs, with pronounced effects by early therapy. Total HIV-1 DNA quantified from bone marrow, lymph nodes and spleen are represented for early treated (n=9), late treated (n= 11) and untreated mice (n=6) (color coding legend in the upper right corner of figure). Statistical analysis was performed by non-parametric Kruskal Wallis test with multiple comparison Dunn's correction. (* $p< 0.05$; *** $p< 0.01$; *** $p < 0.001$; **** $p < 0.0001$)

Immune activation/exhaustion on peripheral CD4⁺ and CD8⁺ T cells is reduced by early ART only

Infection with HIV-1 induces not only loss of CD4 + T cells but also long-term alterations on the activation status of CD4⁺ and CD8⁺ T cells which persist during ART (46,47). Therefore, we assessed T cell phenotypes in the tissues at the end of treatment and corresponding time points for untreated animals by flow cytometry. As seen in the peripheral blood, the relative number of CD4⁺ T cells was significantly increased by ART in the spleen as compared to the untreated group and independently from the timing therapy initiation (supplementary Figure 2, A). PD-1 and TIGIT tended to increase from early treated over late treated to untreated mice (with significant difference between untreated and early treated groups). HLA-DR was not significantly different between the infected groups, but was highest in some of the late treated mice (Fig 4, A-B) and trended to lowest in CD4⁺ and CD8⁺ T cells of early treated animals (figure 4, A-B). Significant differences were observed in PD-1 and TIGIT expression between early versus untreated mice for both CD4⁺ and CD8⁺ T cells (Figure 4, A-B). In addition, we observed a normalization of CD27 expression on CD4 + T cells by early treatment only (Supplementary Figure 2, B) suggesting a maintained T cell differentiation by early ART. These data corroborate observations from peripheral blood in human and highlight the importance of rapid viral suppression in order to preserve the T cell compartment (48).

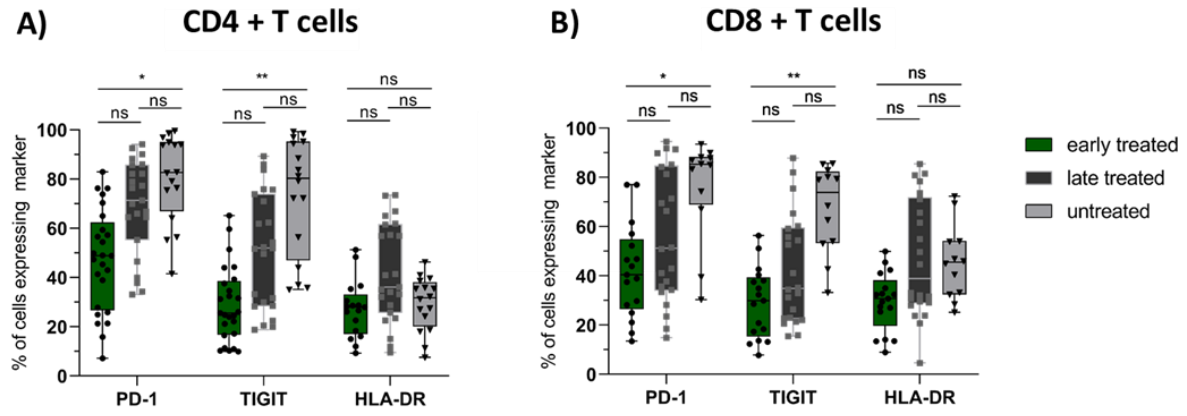


Figure 4. Early treatment significantly lowered HIV-related immune activation on human T cells from the spleen.

Percentage of cells expressing the respective marker on CD4 + T cells (A) and CD8 + T cells (B) extracted from spleen at experimental endpoint (median age of 9 months, 8 weeks total ART duration for both treatment groups) are depicted (early treated (n=17) late treated (n= 23) untreated (n= 16)). Statistical testing by non-parametric Kruskal Wallis test with multiple comparison Dunn's correction. (* p< 0.05. *** p< 0.01 and *** p < 0.001)

$CD32^{+}CD4^{+}$ memory T cells harbor higher amounts of cell assosiated- (CA-) HIV-1 DNA but similar levels of HIV-1 CA-RNA

Next, we investigated the expression pattern of CD32, a proposed marker of the HIV-1 reservoir. Reported technical difficulties to obtain pure $CD32^{+}CD4^{+}$ subsets (31,49), demand a strict gating strategy for $CD4^{+}$ memory T cells (defined as $CD45RA^{-}$). To preclude reported contaminants from B cells or macrophages, CD19 and CD14 were included in the panel as well (Supplementary Figure 3). Using this setting human $CD4^{+}$ T cells contained a $CD32^{dim}$ population with low expression of CD32 in contrast to a $CD32^{bright}$ population expressed in $CD19^{+}$ B cells and doublets (supplementary figure 3, B). Of note, CD32 expression did not differ substantially across various tissues nor between treatment groups (Figure 3, A-D). Nevertheless, in spleen and lymph nodes, early treated-mice tended to have the lowest CD32 expression (< 1 %), with a significant difference in lymph nodes (Figure 5). Importantly we did not find any difference during longitudinal sampling in the spleen (supplementary figure 4, B). Overall, these data showed a rather low but consistent CD32 expression on $CD4^{+}$ memory T cells in the various tissues of different treatment groups.

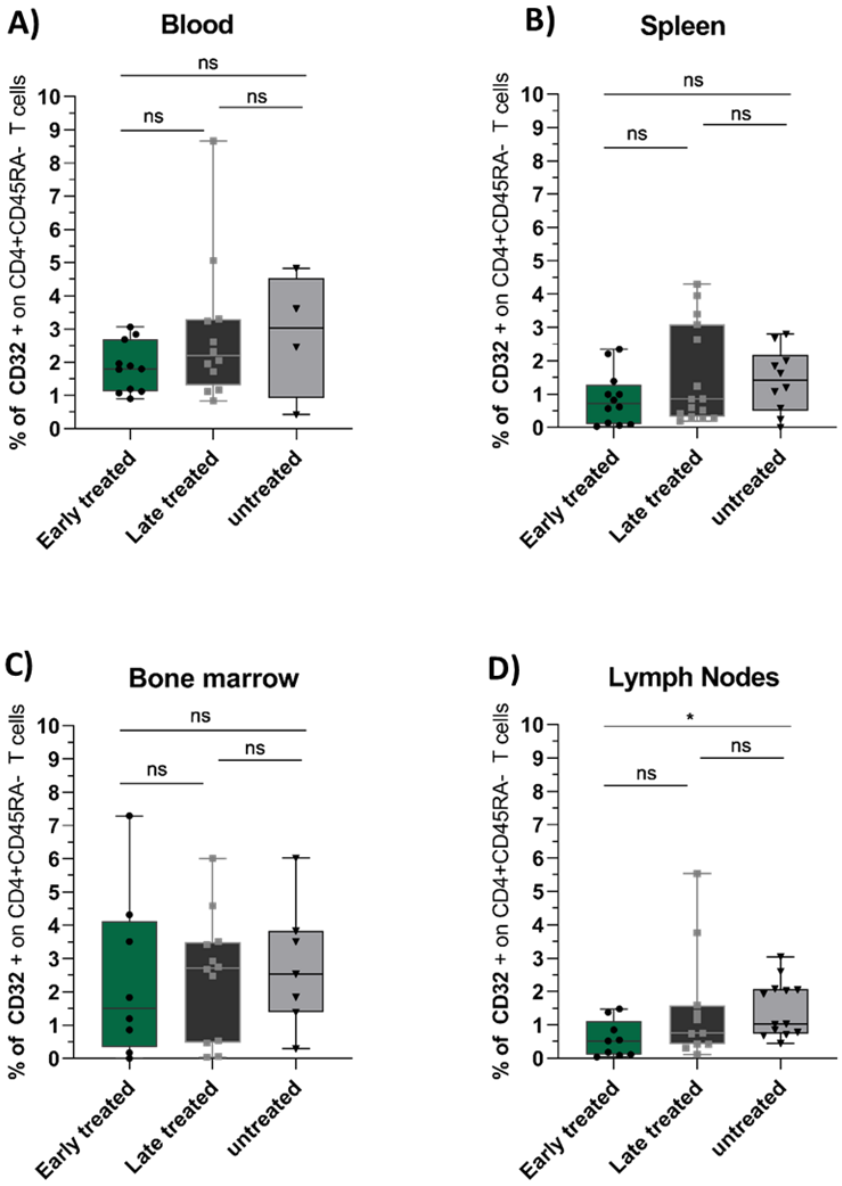


Figure 5. CD32 expression on memory CD4⁺ T cells from different tissues and treatment groups is similar. Expression of CD32 on $CD4^+CD45RA^-$ T cells in blood (A), spleen (B), bone marrow (C) and lymph nodes (D) from flow sorting experiments (early treated (n= 8) late treated (n=12) untreated (n= 4-13)). Statistical testing by non-parametric Kruskal Wallis test with multiple comparison Dunn's correction. (* p< 0.05. *** p< 0.01 and *** p < 0.001)

To determine whether CD32 expressing cells were enriched for total cell associated (CA) HIV-1 DNA or RNA in ART suppressed mice, $CD32^-$ and $CD32^+$ were flow sorted from the $CD4^+$ memory T cells. Sufficient numbers of rare $CD32^+CD4^+$ memory T cells were obtained from the bone marrow and the spleen only. Interestingly, memory $CD32^+CD4^+$ T cells harbored significantly higher levels of HIV-1 DNA as compared to their CD32 negative counterparts (figure 6, A-B) in the spleen (4.35 vs 3.51 median

log_{10} copies of HIV-1 DNA per million cells, respectively, p< 0.01) and in the bone marrow (4.42 vs 3.55 median log_{10} HIV-1 DNA copies per million cells, respectively, p< 0.05). We next quantified cell associated RNA, only few samples from the spleen were containing enough RNA for quantification and we did not detect any differences in the CA-RNA content between the $CD32^+CD4^+$ memory subset and the $CD32^-CD4^+$ in the spleen (Figure 6, C) (42.63 versus 19.46 median copies of CA-RNA per million cells).

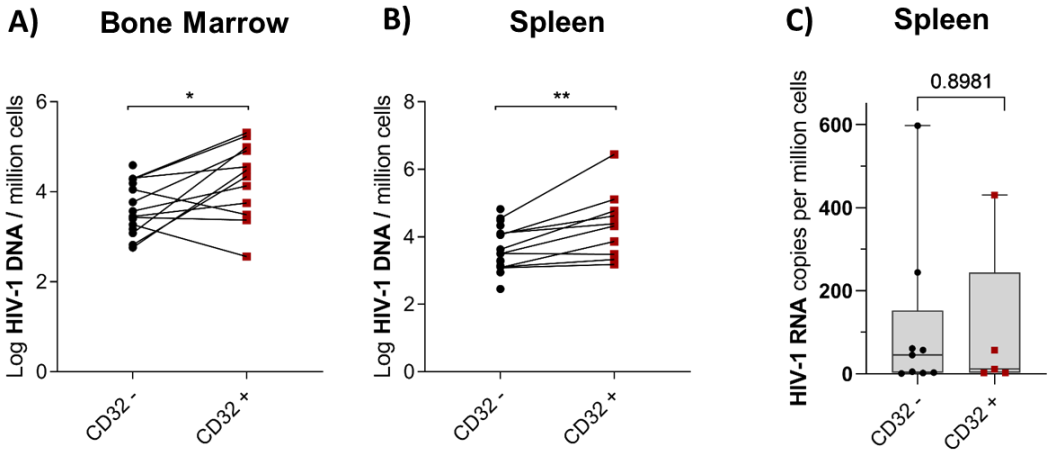


Figure 6. Total HIV-1 DNA is higher in $CD32^+CD4^+$ memory T cells but CA-RNA is not different.
Total HIV-1 DNA from flow sorted $CD3^+CD4^+CD45RA^-$ T cells according to CD32 expression) (gating strategy depicted in supplementary Figure 3) bone marrow (A) and spleen (B) in virally suppressed mice (bone marrow n=11, spleen n= 10). Depicted as aligned dot plots of individual measurements (CD32 - in black circles and CD32 + in red squares). HIV-1 CA-RNA in flow sorted $CD4^+$ memory T cells ($CD32^-$ (n=9) $CD32^+$ (n=5)) (C). Statistical testing was done using non-parametric paired Wilcoxon rank test. Exact p values for HIV-1 DNA: bone marrow 0.0278; spleen 0.0039. (* p< 0.05. *** p< 0.01 and *** p < 0.001)

CD32 is expressed on highly activated memory CD4$^+$ T cells and does not correlate with the HIV total DNA

Next we assessed whether CD32$^+$CD4$^+$ T cells from tissue lymphocytes showed a particular activation/exhaustion profile as recently suggested (50). We observed a significant upregulation of HLA-DR and PD-1 expression but not of the immune checkpoint TIGIT in CD32$^+$CD4$^+$ memory T cells (Figure 7, A - C) as compared to their CD32$^-$ counterpart in both early ($p< 0.05$) and late treated mice ($p< 0.001$). We also measured the expression of the early and late activation activation markers CD69, HLA-DR and CD38 (all combined in one channel) on the flow sorted memory CD32$^+$and CD32$^-$ CD4$^+$ T cells. A median of 80% CD32$^+$CD4$^+$ memory T cells expressed at least one of these activation markers as compared to a median of only 7 % CD32$^-$CD4$^+$ memory T cells (Figure 7, D). Moreover the per cell expression levels (plotted as mean fluorescence intensity or MFI) of CD32 correlated moderately with those three activation markers (Spearman correlation, p value= 0.0004, r=0.6872) (figure 7, E). In contrast, there was no correlation between CD32 expression and the total HIV-1 DNA content with CD4$^+$ T cells (data not shown, p value= 0.5809, r=-0.2167).

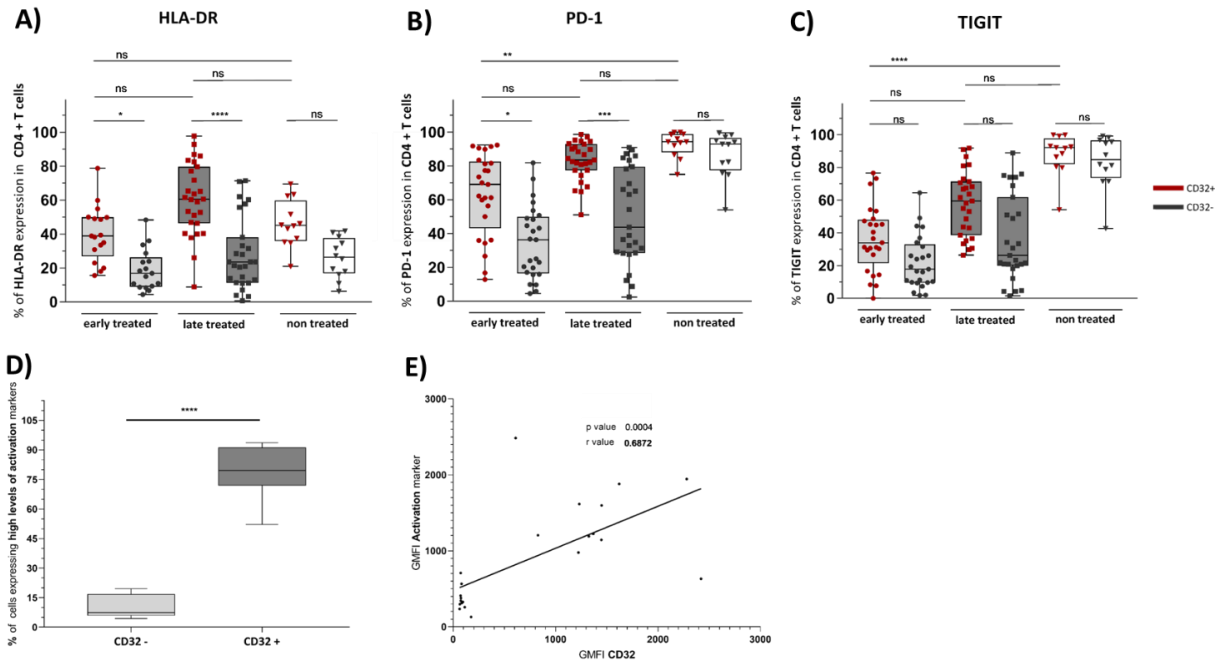


Figure 7. CD32 expression was associated with high expression levels of activation/exhaustion markers and did not correlate with HIV-1 DNA. Expression of HLA-DR (A), PD-1 (B) and TIGIT (C) in CD32 positive and negative CD4$^+$ memory T cells from the spleen. Flow sorted CD4$^+$ memory T cells according to CD32 expression and plotted against the expression level of 3 combined activation markers (HLA-DR, CD38 and CD69) (D). Geometric mean fluorescence intensity (GMFI) of activation markers plotted against the GMFI of CD32. Spearman correlation with fitted linear regression line (p value: 0.0004, r value: 0.6872) (E). Spearman correlation between total HIV-1 DNA and CD32 (F). Statistical testing by non-parametric Mann Whitney or Kruskal Wallis test with multiple comparison Dunn's correction. Two-tailed spearman correlation. (* $p< 0.05$. *** $p< 0.01$ and *** $p < 0.001$)

We performed FlowSom clustering to explore the similarity of cell clusters in longitudinally sampled mice (week 4 and week 8) of early and late treatment regimens. Overall clustering identified five $CD4^+$ T cells population with CD32 expression (figure 8) in both treatment groups. Four of those subsets co-expressed either TIGIT, PD-1, or a combination with HLA-DR (figure 8). Furthermore we observed two clusters statistically reduced (week 4 to week 8) for late treatment (cluster 6 and 9) which coexpress PD-1 or PD-1 and HLA-DR. Early treated mice showed also two different clusters (cluster 21 and 11) which either do not or do show TIGIT and HLA-DR coexpression. These findings substantiate on reduction of immune activation and exhaustion markers by longer treatment duration. Furthermore, the presence of different subsets of CD32 positive cells in the memory CD4 + T cells in treated mice. Taken together clustering showed that CD32 labels mostly an activated portion of the $CD4^+$ T cells, which reduced similarly overtime due to treatment duration as we observed for the general $CD4^+$ T cell population.

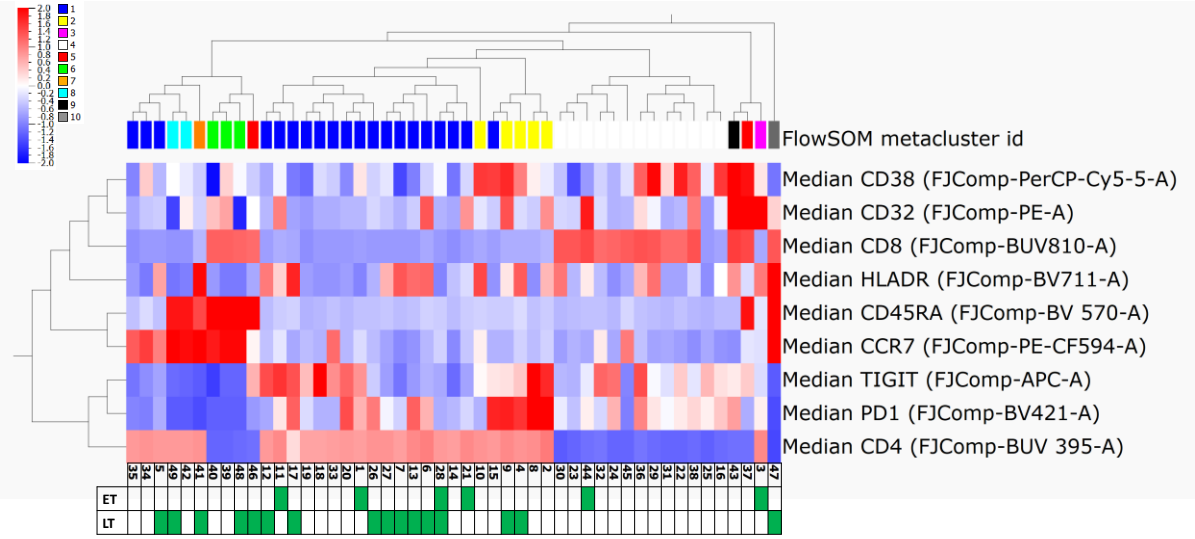


Figure 8. FlowSom analyses highlights different activated clusters of CD32 in memory $CD4^+$ T cells
Hierarchical agglomerative heat map of 49 clusters calculated by FlowSom algorithm using all markers indicated on the right side. Statistical comparison for early treated (ET) or in late treated (LT) mice comparing 4 weeks and 8 weeks of treatment within each group ($p< 0.05$ colored in green below the heat map).

Memory $CD32^{-}CD4^{+}$ T cells contain most of the translation competent virus

Finally we sought to answer whether $CD4^{+}$ $CD32^{+}$ memory T cells contained translation-competent HIV-1. To this end we assessed P24 expression of mononuclear cells isolated in a subset of HIV-infected viremic mice as well as in ART virally suppressed mice (late treatment) after PMA activation. Albeit $CD32^{+}CD4^{+}$ T memory cells contained significantly more HIV-1 DNA, most of the P24 producing cells were detected in the CD32 negative fraction in both vireamic and virally suppressed mice in the spleen (figure 8, A) and in the bone marrow (data not shown). Interestingly within $CD32^{+}CD4^{+}$ memory T cells there was a higher frequency of P24 positive cells as compared to $CD32^{-}CD4^{+}$ memory T cells pointing to a relative enrichment (figure 8, B). Furthermore we find higher levels of activation/exhaustion markers on $CD32^{+}CD4^{+}$ memory T cells producing P24 than their $CD32^{-}$ counterpart which was significant for PD1 only (figure 8, C).

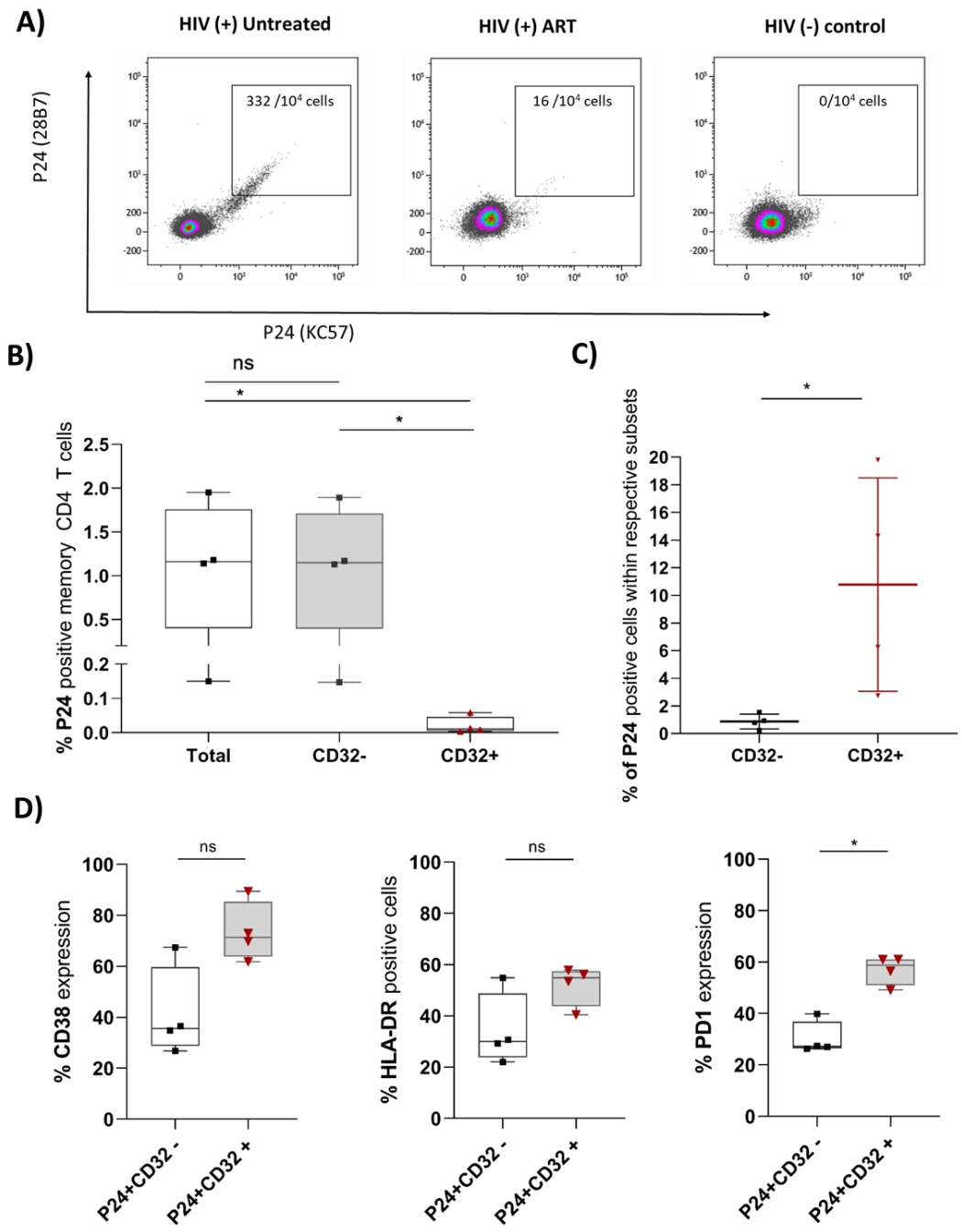


Figure 9: The productive virus is predominant in CD32$^-$CD4$^+$ memory T cells and not in the CD32$^+$ fraction. Representative FACS plots of P24 staining in an untreated viremic mouse after 5 weeks of HIV infection, and one ART-treated mouse and one non-infected control mouse after PMA reactivation (A) in the spleen. Intracellular P24 staining after PMA/ionomycin activation in ART treated animals (n=4) revealed that the highest proportion of productive cells is CD32$^-$ and not CD32 $^+$ (B). The frequency of P24 producing cells is higher within CD32$^+$CD4$^+$ memory T cells than CD32$^-$ cells (C). Expression of HLA-DR, CD38 and PD-1 at the surface of the reactivated cells producing P24 from graph B (D). Statistics by non-parametric testing. (* p< 0.05. *** p< 0.01 and *** p < 0.001)

3.5 DISCUSSION

HIV-1 persists in a pool of latently infected CD4+ T cells identified as the main barrier to HIV cure (51). This reservoir has been determined by early studies to be mainly localized in $CD4^+$ resting T cell memory subsets but recent evidence has substantiated on its wide spread distribution over various compartments of $CD4^+$ T cells (16,20,21,52,53). A number of strategies aiming at reducing the size of the HIV reservoir are currently under investigation. Tailored interventions ideally demand biomarkers to identify the cells containing replication-competent virus. Recently CD32 was proposed as a putative marker but has been controversially debated ever since (54,55). The present study scrutinized the relationship between the timing of ART initiation, T cell immune activation/exhaustion, and reservoir characteristics with regard to CD32 expression in tissues of humanized mice.

We validated the humanized mice model of HIV-1 latency showing that the time to viral suppression was shortened by early ART treatment as compared to late treatment. In agreement with Grinsztejn et al. (56) we report here comparable relative numbers of CD4 + T cells depletion between early and late treated mice. Multiple studies have reported the benefits of early ART on immune activation (57–59). We observed a consistent decline associated with ART duration on the T cell activation marker HLA-DR in both $CD4^+$ T cells and $CD8^+$ T cells as reported previously by others (58,60). In line with our observation early treatment further decreased T cell activation but did not normalize it to levels of uninfected controls (58). Persistent chronic infections as HIV-1 drive T-cell exhaustion reflected by the expression of immune checkpoint such as PD-1 and TIGIT (61,62). During ART the reduction in PD-1 expression on $CD4^+$ and $CD8^+$ T cells coinicides with decreasing plasma VL (63). Similarly we observed that PD-1 and TIGIT expression were significantly decreased by early ART only. In summary these findings showed a convincing recaputilation of main aspects of HIV-1 pathogenesis and ART-induced suppression in humanized mice.

It is accepted that early ART effectively reduces the size of viral reservoirs and preserves the functionality of $CD8^+$ T cells, two conditions that might allow some individuals to achieve functional HIV cure (64). Our findings support a time dependent escalation of the HIV-1 reservoir spread (65,66). Conversely the time to viral rebound did not differ between early and late treatment groups although some early treated mice showed only transient viraemia. Several clinical studies have reported similar results and found no prolonged control upon treatment cessation although early ART initiation (6,67). Our results suggest that total HIV-1 DNA, even when sampled in tissues, is not a surrogate marker of

time to viral rebound. However, it has to be taken into account that the used humanized mice model does not develop a mature, antigen specific $CD8^+$ T cell response (68). Therefore it is quite possible that a lack of antiviral immunity leads to a more rapid rebound or more intact proviral sequences during reservoir seeding (69). Taken together these data show that the viral reservoir is already established and functional within one week after infection allowing a rapid viral rebound after treatment interruption.

A number of cellular HIV-1 reservoir markers have been proposed over the last years. Among those the activation and immune checkpoint molecules HLA-DR, PD-1, TIGIT, but also CD32a (25,28,29,70,71). According to the original publication, CD32a had the promising trait of containing the replication-competent but also to show an enrichment of about 10^3 in copies of total HIV-1 DNA (29). These results have been challenged by others reporting either no (30,31), or only marginal enrichment (34,49), for both HIV-1 DNA and replication-competent proviruses. Such divergent findings have been in part attributed to technical tribulations during purification of $CD32^+CD4^+$ T cells (35). We implemented a gating strategy for the flow and sorting analyses allowing a strict exclusion of doublets and $CD19^+$ and $CD14^+$ cells. This enabled the identification of a $CD32^{dim}$ population expressed in a small fraction of $CD4^+$ T cells (Supplementary Figure 4, A). We further focused on CD32 expression in memory $CD4^+$ T cells ($CD45RA^-$) and included both resting and non-resting reservoir sites. Importantly a significant increase of about 0.85 $\log_{10}$ copies of CA-HIV-1 DNA per millions cells in $CD32^+$ memory T cells compared to $CD32^-$ memory T cells of virally suppressed mice was observed. These findings are in agreement with results obtained from human lymph nodes (72) and human rectal tissues (63) but not with data derived from peripheral blood of HIV-infected patients (49,50,73,74). Therefore we assume that this enrichment might be specific to tissues-resident cells and is not reflected in the peripheral blood as debated in the field (35). Despite this enrichment there was no difference of CA-HIV-1 RNA between $CD32^+$ and $CD32^-CD4^+$ memory T cells from the spleen suggesting that CD32 does not label higher HIV-1 transcription in peripheral tissues. These findings are in accordance with previous studies showing only an enrichment for lymph node resident cells (30,72). Importantly when looking at inducible translation-competent virus the majority of P24 expressing cells was found in the $CD32^-$ memory T cell subset in both viremic and ART treated mice. Of note CD32 expressing cells showed relative enrichment of P24 which is in agreement by the higher levels of CA-HIV-1 DNA. However $CD32^+CD4^+$ T cells formed a negligible portion of the total translation competent reservoirs. Mohsen et al. recently reported similar findings in human lymph nodes (73). Ultimately our data clearly supports that CD32 expression is a weak contributor to the total translation competent HIV-1 reservoir.

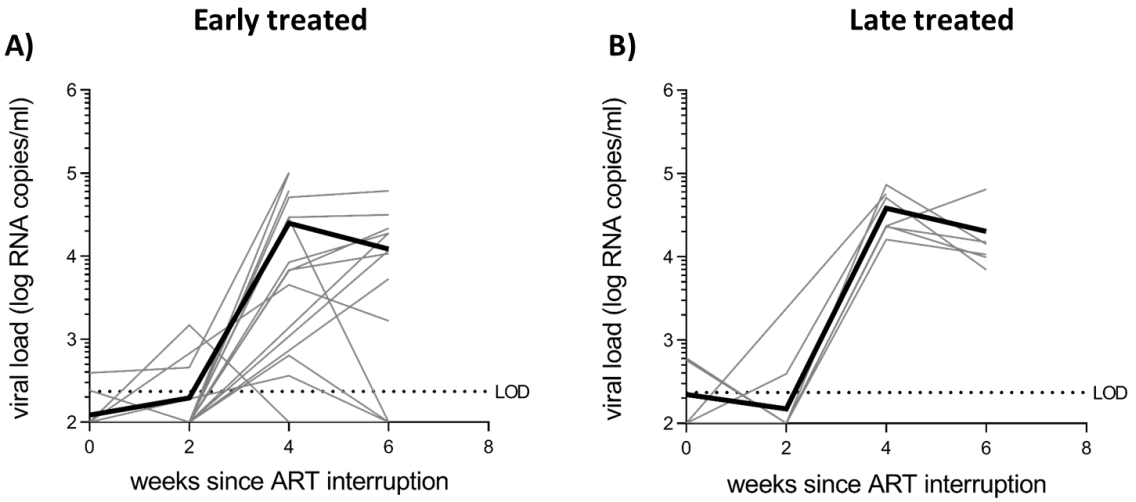
CD32 represents a link between humoral and cellular immunity and is highly expressed on myeloid cells and B cells (35). Of note the functional role of CD32 on $CD4^+$ T cells remains to be precisely understood. It has been suggested that immune complexes might contribute to the continuation of chronic inflammatory responses by enhancing the activation of T cells through CD32 (75). Importantly, the expression of HLA-DR, CD38 and CD69 was strongly increased in $CD4^+CD32^+$ memory T cells from the tissues of ART-treated mice in accordance with previous findings raised in the peripheral cells from HIV-infected patients and healthy controls (76). Using multidimentional reduction analysis, we showed that $CD32^+$ $CD4^+$ memory T cells harboured a high activation/exhaustion pattern and clustered phenotypically with distinct activated memory T cells subsets, especially in $CD4^+$ T cells as compared to $CD8^+$ T cells in mice treated with ART. Therefore a higher activation status could in turn play into preferential infection of $CD4^+CD32^+$ and could explain subsequent enrichment in total HIV DNA. This hypothesis is supported by increased levels of CXCR4 and CCR5 expression in these cells (76,77). Our findings on low unstimulated CA-RNA expression and p24 levels in lymphoid tissues did not substantiate that CD32 label recently infected or re-activated cells. In other studies CD32 expression was associated with persistent HIV-1 transcription in lymphoid tissues (32,77) explained partly by a poor ART penetration. As suggested by others (31), we can not exclude that the P24 expression was released from CD32b + T-B cell doublets localized preferentially in sites with higher HIV infection in these studies since the anti-CD32 antibody does not discriminate between isoforms.

Finally, the clinical relevance of the memory $CD4^+$ $CD32^+$ T cells in the context of long term viral reservoir remains questionnable as they appear highly activated hence rather short lived (32,78). We did not observe any correlation between CD32 expression and CA HIV DNA in the cells isolated from the spleen and the bone marrow, but rather a correlation of CD32 with the co-expression of the activation markers. Infection of HIV-1 primarily occurs in activated $CD4^+$ T cells which can revert from an activated to a resting state forming the long lived memory reservoirs (79,80). Since CD32 was shown to promote immune activation in $CD4^+$ T cells in vitro (75), it is tempting to speculate that CD32 itself could induce and maintain activation leading to preferential vulnerability to infection. However if so this would prevent its role in long lived reservoir. Our data futhermore suggests a time dependent reduction of immune activation during suppressive thearpy on $CD32^+$ subsets similarly to the overall $CD4^+$ T cell compartment. Finally there was a significant upregulation of PD-1 in $CD32^+$ vs $CD32^-$ cells after PMA stimulation suggesting that the reactivated memory $CD4^+$ T cells producing the translation-competent virus were functionally different and linked to a particular subset of CD32+ cells. Interstingly

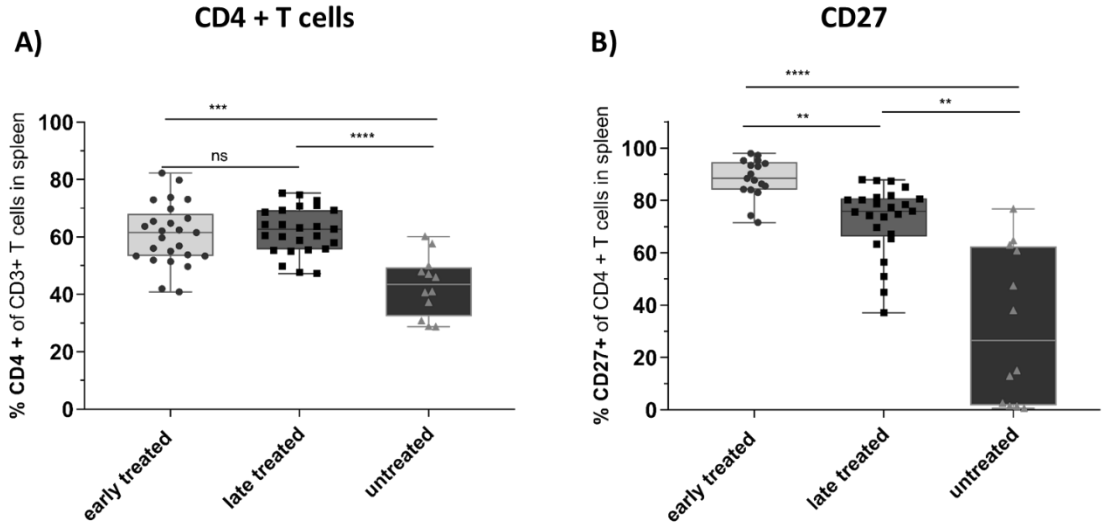
Noto et al. has associated $CD32^{+}PD1^{+}CD4^{+}$ T cells from lymph node with the highest levels of HIV-1 transcription (32). To this end a combination of mutliple markers to identify highly productive HIV-1 reservoirs might be useful.

In conclusion, the present work found tissue resident CD32+CD4+ memory T cells being highly activated subsets increased for total HIV-1 DNA but not for CA-RNA. Despite a relative enrichment, $CD32^{+}$ $CD4^{+}$ T cells form an overall negligible portion of the replication competent reservoirs. Our findings do not support that CD32 is an elusive marker of HIV-1 reservoir but rather of immune activation in tissues. More distinctive markers to target the the productive HIV-1 reservoir are still needed.

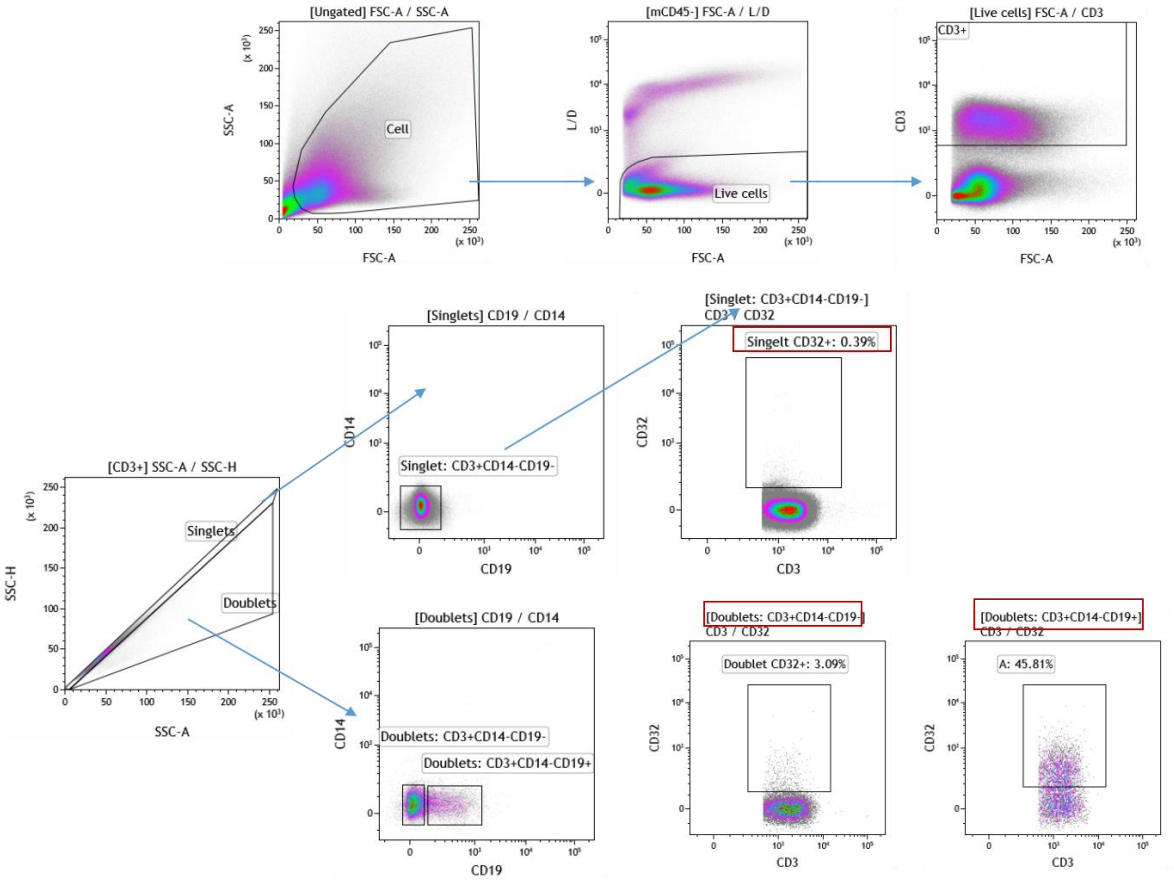
3.6 SUPPLEMENTARY MATERIALS



Supplementary Figure 1. No difference in viral rebound between early and late treatment.
Individual values of HIV-1 RNA copies in log10 scale per ml of blood plasma over time after treatment interruption in light grey lines. (Group mean in bold black line).

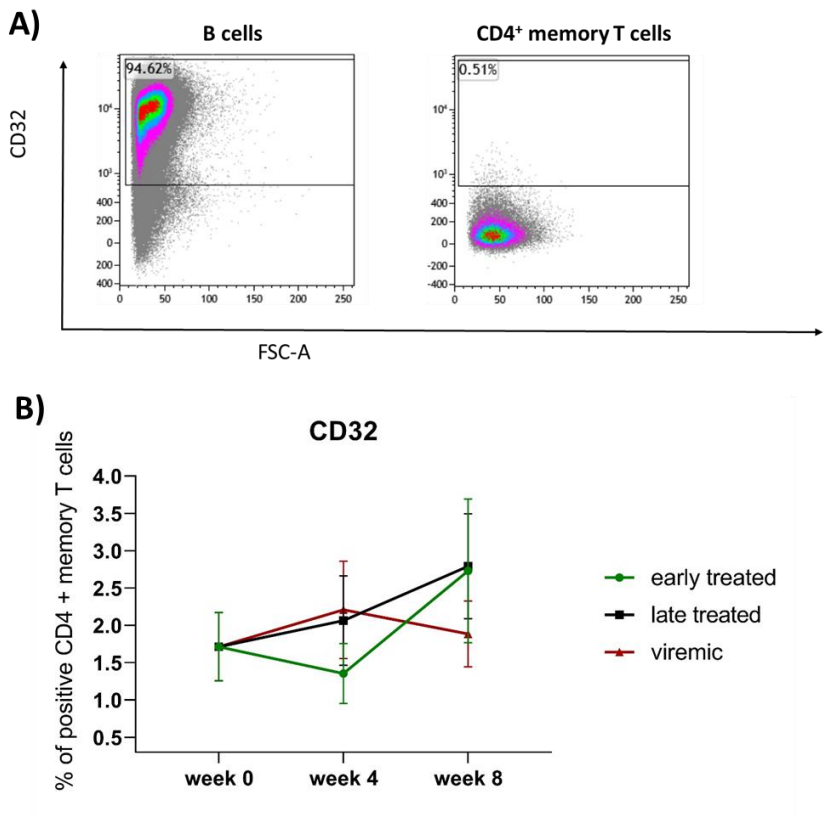


Supplementary Figure 2. Relative percentage of CD4⁺ T cells (A) and CD27 expression in CD4⁺ T cells (B) were preserved by ART in the spleen of humanized mice. Percentage CD4⁺ T cells in spleen (A) and expression of CD27 in CD4⁺ T cells (B). Statistical testing was done by non-parametric Kruskal Wallis test with multiple comparison Dunn's correction.



Supplementary Figure 3. Gating strategy during phenotyping experiments for identification of $CD32^{+}CD4^{+}$ T cells

Following a lineage gating strategy (top layer) we strictly gated on singlet cells and further excluded potential impurities by gating on negative events for CD14 and CD19, leading to a highly pure CD32 ^{dim} T cell population. In contrast we see clear doublet cells of t and B cells (potentially monocytes) leading to a CD32 ^{Bright} population.



Supplementary Figure 4. Longitudinal expression of CD32 on CD4$^+$ memory T cells
Representative FACS plots of CD32 expression on B cells and CD4$^+$ memory T cells (A). CD32 expression on CD4$^+$ memory T cells did not change significantly over time between treatment groups (n=34) (B). Three groups of mice were infected with HIV (n= 34): viremic untreated mice (n= 12), or mice receiving early ART treatment (n= 12) or late treatment (n= 10).

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4 *IN VITRO* VIRAL SUPPRESSIVE CAPACITY CORRELATES WITH IMMUNE CHECKPOINT MARKER EXPRESSION ON PERIPHERAL CD8+ T CELLS IN TREATED HIV POSITIVE PATIENTS

Author's contribution: The following chapter was a common work with the co-first author of this article, Pieter Pannus.

In-vitro viral suppressive capacity correlates with immune checkpoint marker expression on peripheral CD8+ T cells in treated HIV positive patients

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AIDS 2019, 33(3), 387-398

4.1 ABSTRACT

Objective: To determine whether viral suppressive capacity (VSC) of CD8+ T cells can be boosted by stimulation with HIV-1 peptides and whether the ability to control HIV-1 replication correlates with immunological (cytokine production and CD8+ T cell phenotype) and viral reservoir measures (total HIV-1 DNA and cell-associated RNA) in well-treated HIV-infected chronic progressors.

Design: We compared VSC of peripheral CD8+ T cells to cytokine production profile in response to peptide stimulation, detailed phenotype (17-color flow-cytometry), reservoir size (total HIV-1 DNA), basal viral transcription (unspliced cell-associated RNA) and inducible viral transcription (*tat/rev* induced limiting dilution assay) in 36 HIV+ patients on cART and six healthy donors.

Results: We found that the VSC of CD8+ T cells can be increased by prior stimulation with a pool of consensus HIV-1 *gag* peptides in a significant proportion of progressor patients. We also found that VSC after peptide stimulation was correlated with higher expression of immune checkpoint markers on subsets of terminally differentiated effector memory (TEMRA) CD8+ T cells as well as with production of IFN-γ, TNF-α and IL-10. We did not find a correlation between VSC and viral reservoir measures.

Conclusions: These results add to a small body of evidence that the capacity of CD8+ T cells to suppress viral replication is increased after stimulation with HIV-1 peptides. Interestingly, this VSC was correlated with expression of immune checkpoint markers, which are generally considered to be markers of exhaustion. Our findings may guide further investigations into immune phenotypes correlated with viral suppression.

4.2 INTRODUCTION

HIV-1 persists in a reservoir of long-lived latently infected cells [1, 2]. Most HIV-1 infected subjects are dependent on combination anti-retroviral therapy (cART) to suppress viremia [3, 4] ('progressors'), while a small proportion controls viremia without treatment ('elite controllers') or after a period of cART ('post-treatment controllers') [5]. The concept of "functional cure" or "viral remission" is to induce viral control without cART in 'progressors' [6-9].

It is generally accepted that a sufficiently reduced viral reservoir and/or potent cytotoxic T cell (CTL) responses are needed to achieve viral remission [10]. Indeed, lower levels of HIV DNA and cell-associated RNA in peripheral blood mononuclear cells (PBMCs) before structured treatment interruption in chronically infected subjects have been associated with delayed time to viral rebound [11, 12]. Studies comparing the direct ex-vivo CTL potential of elite controllers and classical progressors illustrate the importance of polyfunctionality and viral suppressive capacity (VSC) of CD8+ T cells in the control of viremia [13-15]. Furthermore, a correlation between lower pre-therapy expression of immune checkpoint markers on T cells and delayed viral rebound after treatment interruption was shown in the SPARTAC trial [16].

To reduce the size of the viral reservoir, the shock-and-kill strategy, using latency reversing agents, is one currently attempted approach [17]. On the other hand, inducing potent CTL responses might be necessary and could be achieved by therapeutic vaccination. In this respect, in-vitro models evaluating the VSC of CD8+ T cells could help in the evaluation of vaccine candidates before going into clinical trials.

Viral inhibition assays generally use CD8+ T cells which are either unstimulated or stimulated in a polyclonal, non-specific way. According to most published data, this type of VSC is absent or very low in 'progressors', even if they are treated with cART [14, 15, 18]. There are some indications that VSC can be induced by HIV-specific stimulation in-vitro [19-21], but whether this is the case in all or only some progressors and whether or how this immune function correlates with other immune and viral markers, has not been studied in-depth until now.

Because *gag*-specific immune responses have been associated with protective immunity [22-25], we set up a viral inhibition assay in which patient-derived CD8+ T cells are specifically stimulated with a pool of HIV-1 *gag* peptides before co-culturing them with super-infected autologous CD4+ T cells. We hypothesized that variation in *gag*-stimulated VSC exerted by CD8+ T cells from well-treated progressors will be observed and that it will correlate with some immunological parameters, such as

cytokine production levels or CD8+ T phenotypes, or with viral reservoir measures, such as total HIV DNA and levels of basal and induced viral transcription in CD4+ T cells. Investigating this conceptually important anti-viral function of CD8+ T cells in well-treated progressors and studying its correlation with established immune and viral parameters will provide useful "baseline" knowledge for future immune-based interventions in this large patient population that is to be included in future interventions towards "remission" or "cure".

4.3 Material and Methods

Study subjects and samples

Thirty-six chronic HIV-1 infected (progressor phenotype), asymptomatic, cART-treated, adult patients were recruited at the Institute of Tropical Medicine (ITM) in Antwerp. Inclusion and exclusion criteria are given in supplementary methods. One hundred and fifty mL of EDTA blood was collected from these patients. We also obtained buffy coats from six healthy HIV-seronegative adult controls from the Red Cross Blood Transfusion Center of Mechelen, Belgium. All participants gave written informed consent per the Declaration of Helsinki under Antwerp ITM Review Board-approved protocols (Belgian registration number: B300201526243).

PBMCs were isolated by Ficoll-Hypaque (Axis-Shield, Oslo, Norway) density gradient centrifugation, resuspended in 90% FBS 10% DMSO, kept overnight in Mr. Frosty boxes (Thermo Fischer Scientific, Waltham, MA, USA) at -80°C and stored in liquid nitrogen until use.

Viral inhibition assay

Viral suppressive capacity (VSC) was measured using a viral inhibition assay that was based on a published protocol with significant modifications [20].

For the preparation of CD4+ T target cells, PBMCs were stimulated during seven days at 37°C 7% CO_2 in RPMI 2.5% human serum (HS, A&E Scientific, Belgium), IL-2 (500IU/mL, Gentaur, Kampenhout, Belgium) and anti-human CD3/8 bi-specific monoclonal antibody (1µg/mL, NIH AIDS Reagent Program). For the preparation of stimulated CD8+ T effector cells, PBMCs were incubated during 7 days at 37°C 7% CO_2 in RPMI 2.5% HS and 10µg/mL (81ng/mL per peptide) of an HIV consensus subtype B *gag* peptide pool (NIH AIDS Reagent Program). For the preparation of non-stimulated CD8+ T effector cells, an extra aliquot of PBMCs was thawed one day before the start of CD4+/CD8+ T cell co-culture and rested overnight at 37°C 7% CO_2 in RPMI 2.5% HS.

On day 0, CD4+ T target cells were enriched by negative selection magnetic beads (Miltenyi Biotech, San Diego, CA, USA) from the anti-CD3/8 mAb stimulated PBMCs. Stimulated as well as non-stimulated overnight rested CD8+ T effector cells were enriched from the *gag* peptide pool stimulated PBMCs and overnight rested PBMCs respectively, by negative selection. Cell purities of enriched CD4+ T and CD8+ T cells >90% were confirmed by staining for CD3 (FITC, Clone OKT3), CD4 (PE, clone SK3), CD8a (APC-eFluor, clone SK1) and reading on a BD FACSVerse (Beckton Dickinson, New Jersey, USA).

Enriched CD4+ T cells were incubated with HIV III_B (NIH AIDS Reagent Program) at a multiplicity of infection of 0.001 for 3 hours at 37°C 7% CO_2. Infected target cells were washed three times and resuspended at 10^6 cells/mL in RPMI 2.5% HS 500IU/mL IL-2 and cultured in triplicate in flat bottom 96-well plates (VWR, Leuven, Belgium) at 10^5 cells/well, alone (positive control) or in co-culture with stimulated or rested enriched CD8+ effector T cells at an effector-to-target ratio of 1:1 and 2:1. Culture medium was refreshed at days 2, 6 and 9. The level of HIV-1 p24 antigen in the supernatant was determined at day 13 by in-house p24 ELISA [226]. Log inhibition values were calculated on day 13 as $\log_{10}$(p24 without CD8+ T cells) – $\log_{10}$(p24 with CD8+ T cells).

Quantification of HIV-1 DNA and RNA

Total genomic DNA and cell-associated RNA were isolated from 5 x 10^6 PBMCs each. Total HIV-1 DNA and cell-associated HIV-1 unspliced RNA were measured with the QX200 Droplet Digital PCR platform (Bio-Rad, Hercules, CA, USA) as described previously [27, 28].

Total HIV-1 DNA amounts were normalized to copies per 10^6 PBMCs with the reference gene *RPP30*. For absolute HIV-1 usRNA quantification, normalization of input cDNA was done by quantifying gene expression levels of stably expressed reference genes as described previously [29, 30]. Data analysis was performed with ddpcRquant [31].

Tat/rev induced limiting dilution assay

The *tat/rev* induced limiting dilution assay (TILDA) was performed as described previously by Procopio *et al.* [32].

Cytokine production after peptide stimulation

Supernatant of PBMCs stimulated for seven days with the HIV *gag* peptide pool (81ng/mL per peptide) was tested for type 1 interferon (IFN), T helper (Th)1, Th2 and Th17, including IFN-α, IFN-β, IFN-γ, tumor necrosis factor (TNF)-α as well as the following interleukins (IL): IL-4, IL-5, IL-10, IL-17, IL-22 and IL-27

with Luminex technology on a Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA, USA). All samples were tested undiluted.

Flow-cytometric phenotyping

Phenotyping of CD8+ T cells at baseline and during co-culture was done with two panels. The "cytotoxicity/activation panel" focused on cytotoxicity and activation markers as well as intra-cellular cytokines and included the following markers: CD3, CD4, CD8, CD107a, IL2, CD56, IFN-γ, Granzyme B, CD45RA, HLA-DR, CD38, CCR7, Perforin, CD57, Zombie NIR fixable viability kit. The "immune checkpoint panel" focused on immune checkpoint markers and transcription factors and included the following markers: CD3, CD4, CD8, CD28, CD27, TIM-3, EOMES, CD160, LAG-3, PD1, 2B4, CD45RA, Tbet, CCR7, TIGIT, Zombie NIR fixable viability kit. A detailed list of all used human species reactive antibodies included in the panels above can be found in the supplementary methods.

Staining of PBMCs for flow cytometry was performed using the FOXP3 transcription factor staining buffer set (eBioscience). Briefly, cells were harvested and washed twice in cold PBS 1% FBS. Next, surface stains and near infrared live dead dye were incubated for 30 minutes. Cells were washed twice, fixed and permeabilized with the eBioscience FOXP3 kit. Cells were then stained intracellularly for 30 minutes and washed twice. Samples were acquired on the Fortessa LSR flow cytometer (BD Immunocytometry Systems, San Jose, CA). Data analysis was performed using FlowJo version 10 and Kaluza version 1.5.

Statistical Analysis

Mann-Whitney U tests were used to compare differences in mean VSC, cytokine levels and phenotypic markers between suppressors and non-suppressors. Linear regressions and Spearman rank tests were used to determine correlations between clinical, virologic and immunological parameters. Family-wise corrections of p-values were made using the Bonferroni-Holm's method.

Cluster analysis of flow cytometry phenotypic data

Flow cytometry data was cleaned and compensated with Flow Jo version 10 software (BD, New Jersey, USA). CD8+ T cell populations were concatenated containing the file internal compensation matrix and reimported into the Cytobank environment. Scaling of channels was applied based on single compensation controls for each marker. Cluster analysis was done by running a SPADE (**S**panning tree **p**rogression **a**nalysis of **d**ensity-normalized **e**vents) algorithm on the mother population of total CD8+ T cells. Clustering was based on CD45RA and CCR7 expression.

SPADE results were verified by manual gating. Median fluorescence intensities (MFI) for each cluster were analyzed in GraphPad Prism and fold changes between suppressors and non-suppressors were calculated as ((MFI(Suppressor) – MFI(Non-suppressor)) / MFI(Suppressor). Multiple t-tests were run to statistically compare all markers between both groups. P-values depicted on figures are not corrected for multiple testing.

4.4 RESULTS

Participant Demographics

The demographics of the 36 HIV+ patients recruited in this study are given in Table 1. Patients with a wide range of age, time on therapy, peak viral load and CD4 counts were included in

Table 1. Patient demographics of 36 included HIV+ patients.

Age (years)		CD4 T cell nadir (cells/µL)	
- Range	31.1 – 50.1	- Range	1 – 610
- Median	40.0	- Median	278
Time on cART (months)		Actual CD4 T cell count (cells/µL)	
- Range	18 – 248	- Range	411 – 1504
- Median	86	- Median	664
Peak Viral Load (cps/mL)		Gender	83% male
- Range	5,290 – 1,020,000	Subtype B infected	64%
- Median	244,604	Non-subtype B infected	36%

order to capture the diversity of our clinical cohort. Thirty-one patients had an undetectable HIV viral load (VL), four patients had a detectable VL <50 copies/mL, one patient had a VL of 52 copies/mL.

CD8+ T cell viral suppressive capacity is strongly enhanced after an HIV specific in-vitro peptides timulation in a subset of progressor patients

We measured VSC of non-stimulated and subtype B *gag* peptide-stimulated CD8+ T cells in six healthy HIV negative volunteers and in 36 HIV-1 infected individuals at effector to target ratios (E:T) of 1:1 and 2:1. Our assay proved to be specific for HIV-1 as minimal suppression was observed in all healthy volunteers, with or without peptide stimulation, whereas strong suppression was observed in some HIV-1 infected individuals (Fig. 1A). Based on the comparison with healthy controls, “suppressors” were defined as having a VSC with peptide stimulation exceeding the average VSC of healthy volunteers + three standard deviations, corresponding to 0.34 $\log_{10}$.

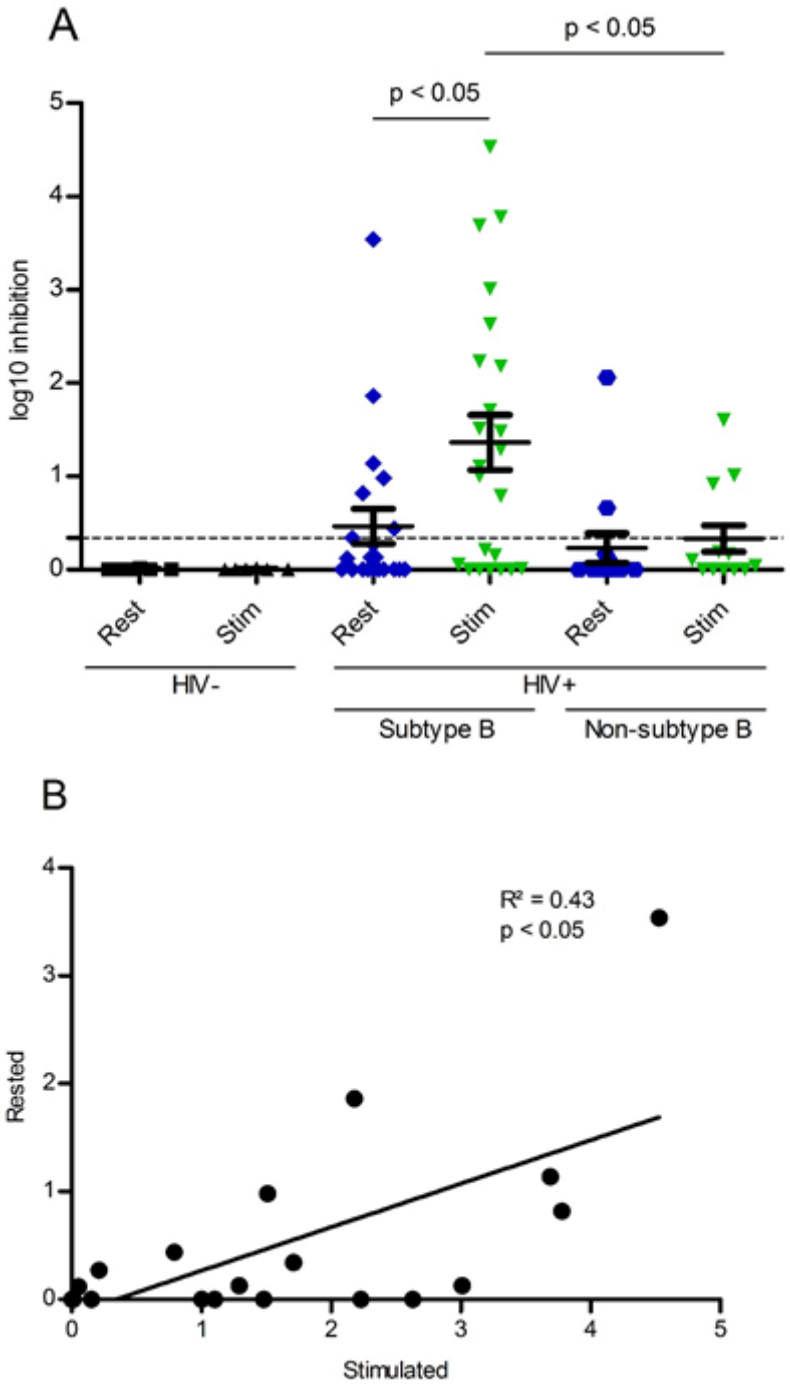


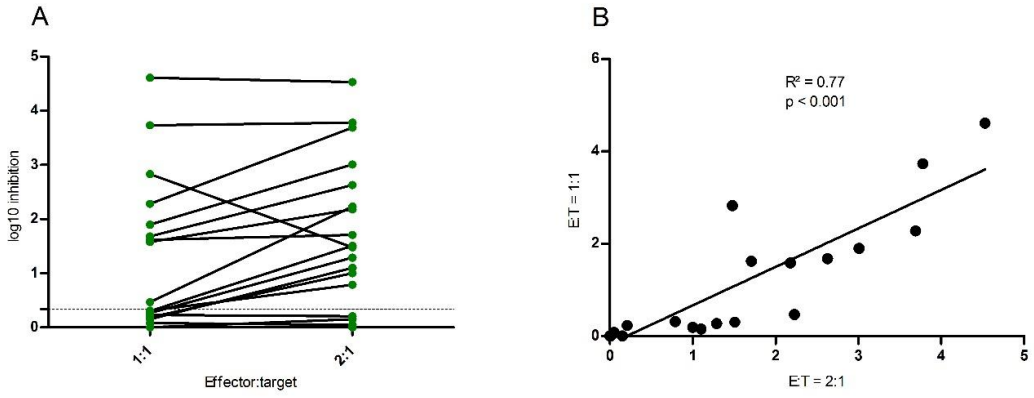
Figure 1. Viral suppressive capacity. **(A)** Log_{10} inhibition values of HIV- people and HIV+ patients (infected with subtype B and non-subtype B HIV-1 strains) with overnight rested (Rest) and 7-day peptide stimulated (Stim) CD8+ T cells as measured in the viral inhibition assay with an E:T of 2:1. Mean with SEM is depicted. The dashed line represents the threshold of 0.36log above which patients are defined as suppressors. **(B)** Correlation between viral suppressive capacity with rested versus 7-day peptide stimulated CD8+ T-cells with an E:T of 2:1.

In subtype B infected individuals, 6 out of 23 (26%) suppressed viral replication without peptide stimulation (mean: 0.47 log_{10}) whereas 14 out of 23 (61%) suppressed viral replication with peptide stimulation (mean: 1.36 log_{10}). The difference in mean response was significant ($p < 0.05$). As expected, no increase in suppression was observed in patients infected with non-subtype B HIV strains after stimulation with the consensus B peptide pool, presumably because these patients have no memory T cells specific for subtype B antigens (Fig. 1A).

Furthermore, we observed a strong correlation ($p < 0.05$) in subtype B infected patients between VSC with and without peptide stimulation (Fig. 1B). As expected, inhibition values were higher at an E:T of 2:1 as compared to 1:1 for most patients (Suppl. Fig. 1A). VSC at both ratios strongly correlated with each other (Suppl. Fig. 1B). On the other hand, VSC did not correlate with current and nadir CD4+ T cell count or peak VL. Nevertheless, in subtype B infected patients VSC with and without peptide stimulation is on average higher in patients with detectable plasma VL as compared to patients with an undetectable VL (1.68 log versus 1.30 log and 0.89 log versus 0.36 log respectively), but these differences are not significant.

HIV viral reservoir measures do not correlate with viral suppressive capacity

Total HIV-1 DNA and usRNA were measured in all 23 subtype B infected patients. HIV-1 DNA was detectable in all patients and ranged from 15 to 859 copies per million PBMCs (mean: 238) (Fig. 2A). Unspliced HIV-1 RNA was detected in 17 out of 23 patients (74%) and ranged from 0.0 to 32.9 copies per million PBMCs (mean: 6.7) (Fig. 2B). These values are consistent with the literature on reservoir size of individuals on cART with suppressed viremia. Total HIV-1 DNA and usRNA did not correlate with VSC or any other studied clinical or immunological parameters.



Supplementary figure 1. (A) Log_{10} inhibition values of HIV+ patients (infected with subtype B HIV-1 strains) as measured in the viral inhibition assay with an E:T of 1:1 and 2:1. **(B)** Linear regression analysis of VSC at E:T of 1:1 versus 2:1.

To further characterize the “functional” viral reservoir, we performed the TILDA assay on 21 available subtype B HIV-1 infected patients. Without PMA/Ionomycin stimulation, no significant transcriptional activity of *tat/*rev RNA was observed (Fig. 2C). With stimulation however, six had detectable amounts of transcription, with values ranging from 55.8 to 313 cells with detectable HIV RNA transcripts per million CD4+ T cells. Therefore pro-viral transcription was inducible with PMA/ionomycin in only a minority (6/21 = 29%) of the patients. VSC did not correlate with induced viral transcription. TILDA inversely correlated with CD4 nadir ($R = -0.67$; $p < 0.05$).

Cytokine Production in Response to Peptide Stimulation

Next, we investigated whether cytokine production in response to HIV peptide stimulation could be predictive of VSC. To this end, we determined cytokine levels in supernatant from PBMCs stimulated for seven days with the HIV-1 *gag* peptide pool, before the start of the CD4+/CD8+ T cell co-culture in 26 available patient samples.

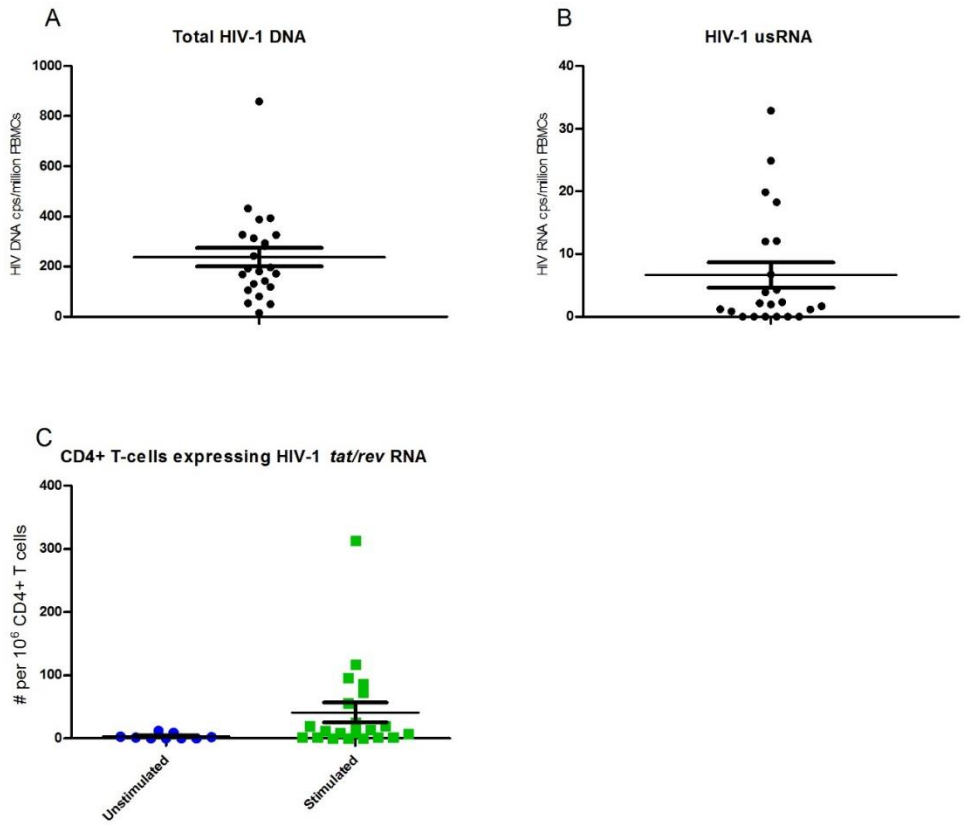


Figure 2. Viral reservoir measures. (A) Total amount of HIV-1 DNA and (B) cell-associated unspliced RNA copies per million PBMCs for all 23 HIV+ subtype B infected patients. Mean with SEM is depicted. (C) Tat/rev induced limiting dilution assay (TILDA) results in number of cells with detectable multiply spliced HIV-1 mRNA per million CD4+ T cells without (N = 10) and with PMA/ionomycin stimulation (N = 21).

When comparing mean cytokine levels, only IFN-γ was produced significantly more in suppressors than in non-suppressors ($p < 0.01$) (Fig. 3A). While TNF-α and IL-10 levels did not differ significantly, their ratio was significantly higher in suppressors than in non-suppressors ($p < 0.05$) (Fig. 3B). Together, these results point towards a correlation between Th1 responses and VSC.

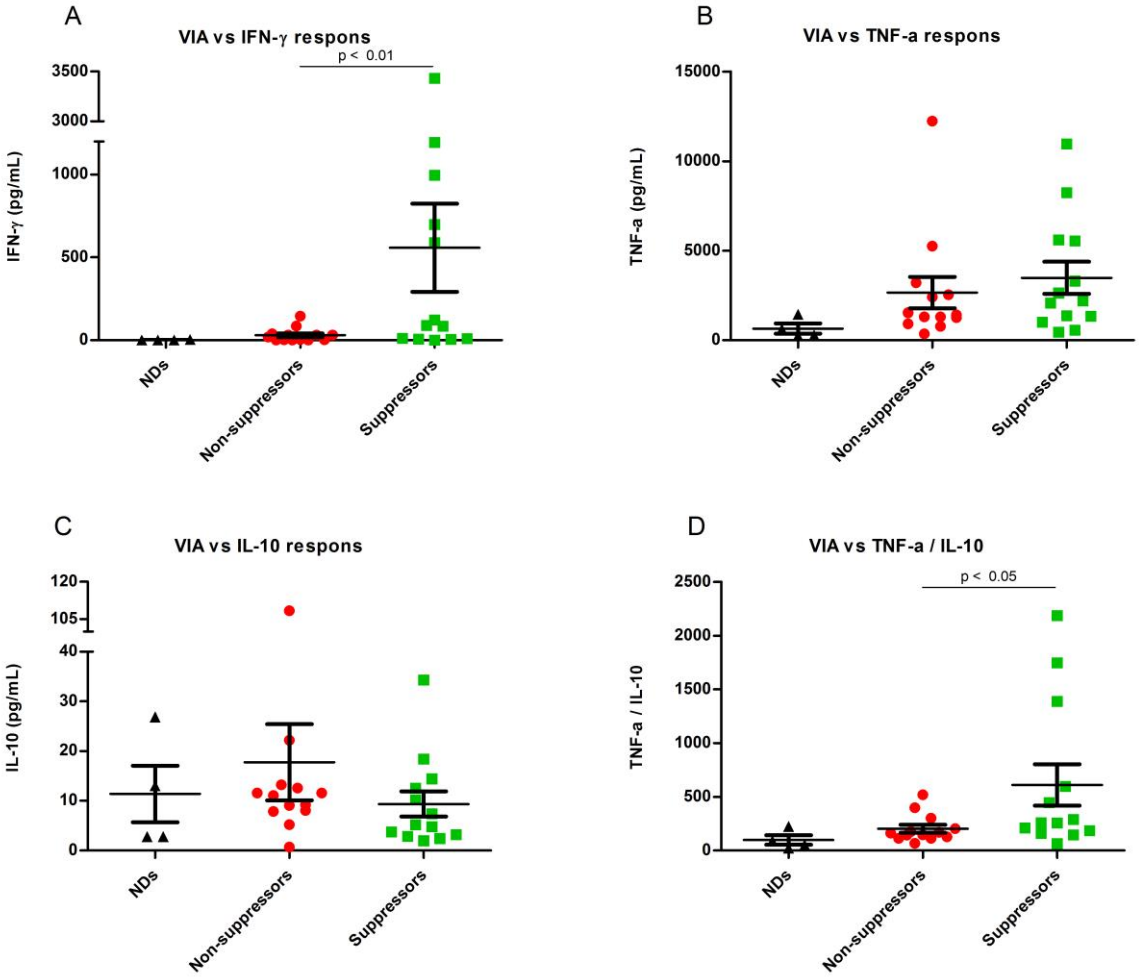


Figure 3. Cytokine production. Cytokine levels in supernatant of PBMCs after 7 days of stimulation with a consensus subtype B HIV-1 *gag* peptide pool from HIV-infected progressors with either a subsequent "suppressor" or "non-suppressor" activity in CD4+/CD8+ co-culture, as compared to HIV-negative subjects (HIV negative donors or ND). (VIA = viral inhibitory activity)

Increased CD160/PD1 co-expression on CD8+ TEMRAs of suppressors at baseline

In order to look for correlates of VSC with so-called 'exhaustion markers' at baseline, we phenotyped non-stimulated, rested CD8+ T cells using the immune check point panel. Interestingly, CD8+ T cells from suppressors only showed a significantly higher CD160/PD1 co-expression in the terminally differentiated effector memory subset (TEMRA), defined as CCR7-CD45RA+, as compared to non-suppressors (Fig. 4A). To identify CD8+ T cell memory subsets

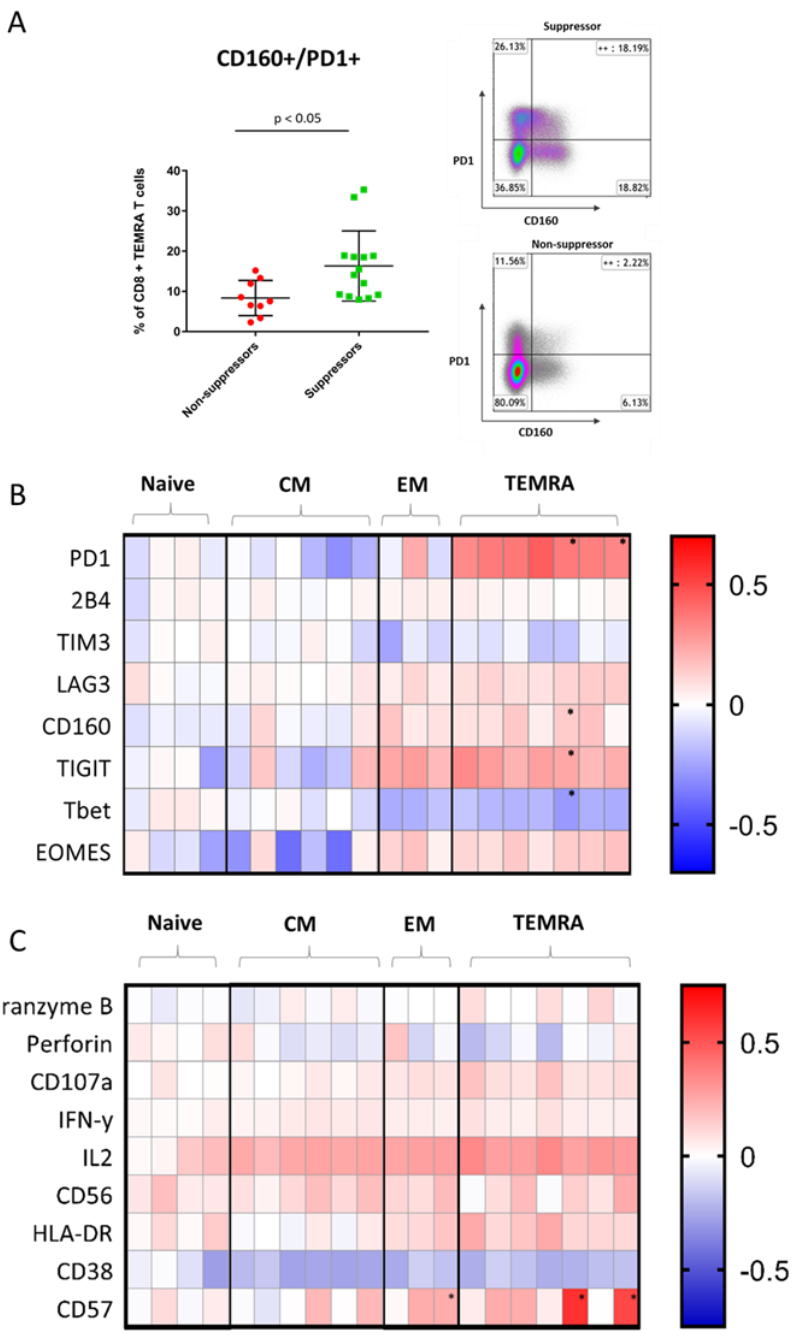


Figure 4. (A) Manual (bivariate gating) analysis showing percentage of PD1/CD160 double positive CCR7-CD45RA+ (TEMRA) CD8+ T cells in suppressors and non-suppressors. Representative flow cytometry plots of suppressors and non-suppressors are given on the right. **(B,C)** SPADE cluster analysis based on CD45RA and CCR7 expression of CD8+ T cells. The heat map depicts for each marker the fold change in MFI of suppressors versus non-suppressors for the 'immune exhaustion' **(B)** panel and the 'cytotoxicity/activation' panel **(C)**. Significant differences ($p < 0.05$) are marked with asterisks (p-values are not adjusted for multiple testing). (CM = central memory; EM = effector memory; TEMRA = terminally differentiated effector memory T cells)

with different expression patterns of exhaustion markers between suppressors and non-suppressors, we ran a SPADE analysis based on CD45RA and CCR7 expression (Fig. 4B). Four, six, three and seven subsets were identified in the naïve, central memory (CM), effector memory (EM) and TEMRA CD8+ T cells respectively (Fig. 4B). The comparison of suppressors versus non-suppressors identified a cluster with significantly higher PD1/CD160/TIGIT co-expression but lower Tbet expression in suppressors. Another cluster showed significantly higher expression of PD1 only. These differences were however only significant without Bonferroni-Holm's corrections of p-values.

Increased CD57 expression on CD8+ T cell memory subsets of suppressors after peptide stimulation

In order to look for correlates of VSC with activation and cytotoxicity markers, we phenotyped CD8+ T cells after 6 hours of stimulation with an HIV-1 consensus B *gag* peptide pool. Using the SPADE cluster analysis described above, we observed significantly higher CD57 expression (without Bonferroni-Holm's correction) in one EM and two TEMRA subpopulations (Fig. 4C) in suppressors. No higher expression was observed for IFN-γ, IL-2, granzyme, perforin or CD107a between suppressors and non-suppressors among the memory subsets after six hours of peptide stimulation.

HLA-DR expression on CD8+ T cells of suppressors is increased early in co-culture

Finally, we phenotyped CD8+ T cells in a small available subset of six patients 18 and 42 hours after the start of co-culture to assess their activation and cytotoxicity phenotype. We found that suppressors had a higher ($p < 0.13$) HLA-DR expression 18 and 42 hours after the start of co-culture as compared to non-suppressors (Fig. 5A). There were no differences in expression of IL-2, granzyme B and perforin between the two groups. Furthermore a slightly higher but non-significant IFN-γ response in suppressors was observed after 42 hours of co-culture (Fig. 5B).

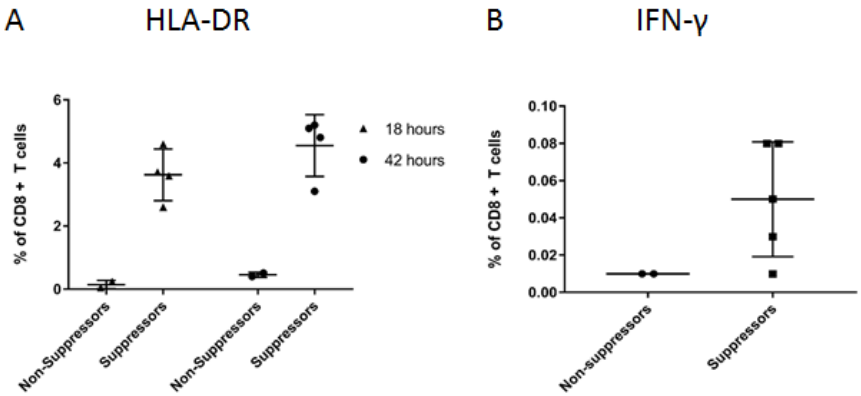


Figure 5. (A) Percentage of HLA-DR positive CD8+ T cells 18 and 42 hours after the start of CD4+ T cell/CD8+ T cell co-culture in suppressors and non-suppressors. **(B)** Percentage of IFN-γ positive CD8+ T cells 42 hours after the start of CD4+ T cell/CD8+ T cell co-culture in suppressors and non-suppressors.

4.5 DISCUSSION

A better understanding of the anti-viral activity of CD8+ T cells is crucial in the search for a functional cure for HIV. In our study we show that in-vitro VSC of CD8+ T cells from a proportion of progressors under cART can be induced with an HIV specific peptide stimulation. While this induced VSC did not correlate with any clinical parameters or viral reservoir measures, we did observe a correlation with IFN-γ production by PBMCs in response to *gag* peptide stimulation. Importantly, we identified specific subsets of TEMRA CD8+ T cells with higher PD1+CD160+ co-expression at baseline, higher CD57 expression after peptide stimulation and higher HLA-DR expression during co-culture with infected CD4+ T cells in patients with higher VSC.

We presume that HIV specific memory CD8+ T cells are expanded during the seven day peptide stimulation and revert from a resting to a more functional effector phenotype, thereby increasing VSC. This idea of specific expansion, rather than the induction of new or non-specific responses is further supported by (a) the strong correlation between VSC of rested versus peptide stimulated CD8+ T cells, (b) the very low background of VSC in healthy controls and (c) the failure to induce VSC in HIV non-subtype B infected patients (Fig.1).

VSC has been described to correlate with plasma viral load [14, 33], suggesting the importance of antigen exposure to keep up cellular anti-HIV immunity. The five patients with low but detectable VLs did have higher VSC with and without peptide stimulation as compared to the patients with undetectable VLs, although this difference was not significant. Similarly, we hypothesized that reservoir size and on-going viral transcription, presumably resulting in antigen presentation and boosting of the immune system, could correlate with CD8+ T cell VSC. Nevertheless, we did not find any correlations with HIV-1 DNA or RNA. It is possible however that the small range in unspliced cell-associated RNA levels in our cohort [IQR: 0.2 – 10.7 cps per million PBMCs] did not allow to observe significant differences in VSC.

Several groups have shown the importance of polyfunctionality and the lack of correlation between VSC and IFN-γ responses of CD8+ T cells [23, 34, 35]. Nevertheless, we observed that the amount of IFN-γ produced in response to an HIV peptide stimulation accumulated over a period of seven days in the supernatant significantly correlated with subsequent VSC (Fig. 3A, $p < 0.01$). Suppressors also had a significantly higher ratio of TNF-α/IL-10 production (Fig. 3D, $p < 0.05$).

Phenotypic data demonstrated higher numbers of PD1+CD160+ double positive CD8+ TEMRA T cells in suppressors. Expression of such immune checkpoint (IC) markers has been linked to T cell exhaustion in the context of chronic disease with sustained antigen exposure, resulting in a gradual loss of effector

functions [36-41]. TEMRA cells on the other hand are also linked to chronic antigen exposure and make up an aged subset of EM cells, characterized by a decreased proliferation potential but strong effector capacities [42]. While the observed positive correlation with VSC therefore seems paradoxical, the following points need to be taken into consideration.

Earlier studies have shown that CD8+ T cells remain responsive despite chronic infection [43, 44]. In lymphocytic choriomeningitis virus infection phenotypically exhausted memory CD8+ T cells control viral infection despite continuous expression of PD1 [45]. During untreated HIV-1 infection increasing viral loads correlate with declining numbers of polyfunctional CD8+ T cells and increased expression of IC markers [13, 46, 47]. During cART the decay of PD1 on T cells coincides with the decline of immune activation and viral load but are never restored to levels of HIV negative individuals [46, 48]. Expression of IC markers has been reported on CD8+ T cells of ECs, suggesting residual viral replication rather than dysfunctionality of the immune response [49]. In addition, ECs harbor highly effective cytolytic subsets in CD8+ T cells expressing CD160 and 2B4 [50]. Taken together, this data supports the idea that a range of exhausted states may exist within functional effector subsets. Accordingly, suppressors were shown to have a larger subset of PD1+CD160+ CD8+ T cells of the TEMRA compartment as compared to non-suppressors, possibly representing more functional and cytolytic cells able to suppress viral replication.

Furthermore, a higher CD57 expression was observed in EM and TEMRA subsets of suppressors as compared to non-suppressors. These respective subsets have typically two profiles: a highly cytotoxic and a poorly replicative one [51, 52]. In addition, CD57 was previously suggested as a marker of proliferative history, and more recently as a maturation marker for cytotoxic HIV specific CD8+ T cells with a more terminally differentiated phenotype in ECs [52-54]. Together, these findings are suggestive of terminal differentiation as a key factor for a potent anti-viral response [55]. Finally, a higher HLA-DR expression in CD8+ T cells was observed in suppressors during co-culture with autologous CD4+ T cells, suggesting that a distinct activation state of fully differentiated memory subsets in suppressors might explain VSC.

While we have identified a phenotypical difference between CD8+ T cells from patients with and without in-vitro viral suppression, further research is needed to determine whether these distinct subsets are in fact responsible for suppressing viral replication.

4.6 REFERENCES SECTION 4

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4.7 SUPPLEMENTARY METHODS

Inclusion criteria for HIV-1 infected subjects:

- Treated with conventional cART.
- Present a routinely "undetectable" plasma VL (< 20 copies/ml) for >6 months.
- They should be in good general condition.
- They should present with actual CD4 T cell count > 300/μL.
- Adult men and women are eligible, but women should not be pregnant.
- Informed consent must be given.

Details of the human species reactive antibodies included in the "cytotoxicity/activation" and "immune checkpoint" panels:

Cytotoxicity/activation panel: BD Biosciences (San Jose, CA): CD3 (BUV496, clone UCTH1); CD4 (BUV395, clone SK3); CD8 (BUV805, clone SK1); CD107a (BV421, clone H4A3); IL2 (BV605; clone 5344.111); CD56 (BV786; clone NCAM16.2); IFN-γ (FITC, clone 4S.B3); Granzyme B (PE, clone GB11). Biolegend (San Diego, CA): CD45RA (Pacific Blue, clone HI100); HLA-DR (BV510, clone L243); CD38 (PerCp/Cy5.5, clone HIT2); CCR7 (PECF594; clone 6043H7); Perforin (PE-Cy7, clone BD48); CD57 (APC, clone HCD57); Zombie NIR fixable viability kit (APC-Cy7).

Immune checkpoint panel: BD Biosciences (San Jose, CA): CD3 (BUV496, clone UCTH1); CD4 (BUV395, clone SK3); CD8 (BUV805, clone SK1); CD28 (BV605, clone 28.2); CD27 (Bv650, clone MT271); TIM-3 (BV711, clone 7D3); Invitrogen (Waltham, MA): EOMES (PE-Cy7, clone WD1928). eBioscience (Waltham, MA, USA): CD160 (FITC, BY55); LAG-3 (efluor 710, clone 3DS223). Biolegend (San Diego, CA): PD1 (BV421, clone EH12.1); 2B4 (Pacific Blue, clone C1.7); CD45RA (Bv786, clone HI100); Tbet (PE, clone 4B10), CCR7 (PECF594; clone 6043H7); TIGIT (APC, clone A15153G); Zombie NIR fixable viability kit (APC-Cy7).

Acknowledgments

We would like to thank Maartje Van Frankenhuijsen, Odin Goovaerts and Meryam Krit from the ITM in Antwerp as well as Karen Vervisch, Maja Kiselinova and Eva Malatinkova from Ghent University for their help in the clinic and laboratory. We would like to thank the Blood Transfusion Center of The Red Cross in Mechelen for providing the buffy coats of healthy donors and the NIH AIDS Reagent Program for providing the HIV-1 Consensus B Gag peptide pool and anti-human CD3/8 bi-specific monoclonal antibodies.

5 POLYVALENT GAG PEPTIDE STIMULATION INCREASED VIRAL SUPPRESSION *IN VITRO* ASSOCIATED WITH THE EMERGENCE OF CYTOTOXIC CD8 + T CELL SUBSETS EXPRESSING CXCR5 IN HIV-1 ELITE CONTROLLERS

Author's contribution: The following chapter is entirely the authors work except the measurements of total HIV-1 DNA by droplet digital PCR, which were obtained in the HIV CURE research center at Ghent (Belgium).

Polyvalent Gag peptide stimulation increased viral suppression *in vitro* associated with the emergence of cytotoxic CD8 + T cell subsets expressing CXCR5 in HIV-1 elite controllers

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Manuscript will be finalized and submitted after some additional analysis on coculture data.

5.1 ABSTRACT

Antiretroviral therapy (ART) is not curative as HIV-1 persists in long-lived reservoirs forcing patients to life-long drug adherence with possible side effects. To overcome these limitations strategies of functional cure aim at ART free viral remission. In this study, we sought to identify subsets of anti-viral CD8 + T cell immunity linked to long-term control of HIV-1 infection.

Here we analyzed PBMCs from two groups of HIV controllers and ART suppressed progressors for *in vitro* viral suppressive capacity (VSC) at baseline and after peptide stimulation. Functional properties and phenotypes of CD8 + T cells were assessed by IFN-γ ELISPOT and eighteen color flow cytometry respectively.

HIV controllers show significantly increased VSC at baseline as well as after peptide stimulation. IFN-γ secretion and the proliferation marker Ki67 positively correlated with VSC. Moreover, the detailed phenotype of three distinct multifunctional CD8 + T cell subsets was a clear feature of elite control only.

Our results underline the importance of HV specific CD8 + T cell responses during natural control. Especially the role of CXCR5 expressing cytotoxic subsets emphasize surveillance in sites of reservoir persistence and demand further study.

5.2 INTRODUCTION

Antiretroviral therapy (ART) halts progression to AIDS by suppressing viral replication but is not curative, because HIV-1 persists in long-lived latent reservoirs (1–3). Interestingly a small group of HIV-1 infected patients succeeds in maintaining low plasma viral loads (pVL) over many years in the absence of therapy. They are regarded as the role model for a functional cure, which refers to HIV remission without ART. HIV controllers are subdivided according to their level of viremia into elite controllers (EC), with pVL < 20 copies, and viremic controllers (VC), with pVL ≤ 2000 copies (4). The association of EC with certain HLA-I alleles is one of the strongest evidence supporting an involvement of anti-viral CD8+ T cells (5–7). An abundance of literature has characterized the anti-HIV-1 CD8 + T cell response in EC displaying greater cytotoxicicity (8,9), higher proliferation (10,11) and polyfunctionality (12,13). There is strong evidence that specificity, quality and broadness are all key determinants of efficient HIV-1- specific CD8+ T-cell mediated responses (14). Also do a number of functional studies substantiate that protection against HIV-1 is more related to the quality (such as polyfunctionality) of the T cell response than to its magnitude (15). Of note higher frequencies of early T cell responses preferentially targeting Gag specific CD8+ T cell responses are predictive of low viral set point and control of infection and found to be dominant in EC (16–18). Among other aspects, targeting Gag constraints immune escape as it comes with high costs in viral fitness when mutated (19,20). Therefore eliciting polyclonal T cell responses targeting conserved epitopes in Gag is a major objective in HIV vaccinology (21).

HIV controllers are an heterogeneous population. Potent anti-HIV CD8+ T cell responses are not restricted to HIV controllers carrying protective HLA alleles. Moreover very weak HIV-1-specific T cell responses can be found in HIV controllers while non-HIV controllers may present with such favorable genetic background (22,23). Taken together, these finding indicate that additionnal factors may contribute to viral to achieve sustained viral control (24).

Direct evidence for viral suppressive capacity (VSC) in vitro is derived from the viral inhibition assay(VIA) which measure viral outgrowth in CD4+ T cells in the presence of CD8+ T cells. Several studies highlighted improved VSC in EC compared to progressors (9,25,26). The breadth of Gag CD8+ T cell responses were found to be associated with higher VSC and reduced viral loads in vivo (27,28) Interestingly low VSC predicts CD4+ T cell decline in ART-untreated patients (29). We recently reported that VSC after peptide stimulation was correlated in ART-treated patients with higher expression of immune checkpoint markers on subsets of terminally differentiated effector memory (TEMRA) CD8+ T

cells producing higher level of IFN-γ, TNF-α and IL-10 (30). Understanding which CD8+ T-cell subsets are most effective in viral suppression is still needed.

Secondary lymphoid organs constitute the major anatomical sites of HIV persistence during ART (31). Recent studies highlighted the importance of lymph node resident follicular CD4 + T helper cells for ongoing viral replication correlating with the presence CD8 + T cell (32). Further data from SIV infection suggest a functional role of B-cell follicle homing CD8 + T cells (CXCR5+) that could contribute to control SIV infection (33). To what degree CXCR5 + CD8 + T cells are present in EC and which functional phenotype they have in HIV-1 infection remains to be clarified.

To this end, we performed in-depth characterization of the CD8+T cell responses of HIV controllers as compared to ART-treated patients using the VIA after expansion of Gag-specific memory responses. We identified specific phenotype of polyfunctional subsets associated with strong CD8 + T-cell responses. For the first time, we showed the emergence of CXCR5 cytotoxic subsets associated with VSC in elite controllers that might target the productive HIV reservoir upon reactivation.

5.3 MATERIAL AND METHODS

Study design and participants

A total of 56 patients were recruited in the Belgian clinical AIDS Reference Centers of Antwerp (Institute of tropical medicine), Brussels (Centre Hospitalière Universitaire Sint Pierre and Universitair Ziekenhuis Brussels), Liège (Centre Hospitalière Universitaire) and Hasselt (Jessa Ziekenhuis). Inclusion criteria defined elite controller (EC) as being treatment naïve, pVL ≤ 20 copies/ml with a duration ≥ 12 months; viral controller (VC) as treatment naïve, pVL 20-2000 copies/ml with a duration ≥ 12 months; ART as patients under treatment, pVL ≤ 20 copies/ml for ≥ 24 months. The numbers of patients in the different categories were as follows: EC (n=15), VC (n=11), ART (n=30). Patients signed informed consent for longitudinal blood donation of 100ml every 6 months over a period of 3 years. The study was approved by each local ethical committee and received the ethical approval from the university hospital of Antwerp (Belgium) under the registration number: B300201731330 as a multicenter Belgian cohort called as PhenoCure In addition anonymised blood samples from healthy donors (HD) (seronegative for HIV, HBV and HCV) were collected from the red cross in Luxembourg as controls for certain immune assays.

Blood was processed within a maximum of 6 hours to maintain functionality of PBMCs. Whole blood separation was done by gradient centrifugation using lymphoprep™ (Stemcell technologies) and LeucoSEP™ (Greiner Bio-One) tubes according to harmonized standard operating procedures across centers. Isolated PBMCs were cryopreserved in commercially available medium (Cryostor, CS10, Stemcell technologies, Vancouver, Canada) in liquid nitrogen (-196°C), plasma and whole blood samples at -80°C untill analysis

Viral inhibtion assay (VIA):

The viral inhibition assay was set-up as described previously with significant modifications (34). CD4+ T cells and CD8 + T cells were prepared and isolated from freshly thawed PBMCs as following :
Preparation of CD4+ T traget cells: freshly thawed PBMCs were stimulated with an anti-human CD3/CD8 bi-specific monoclonal antibody (1µg/ml, NIH AIDS Reagent Program) and cultured during 7 days in R10 medium composed of Rosewell Park Memorial Institute (RPMI) medium with 10% fetal bovine serum (FBS), L-Glutamine (0,4 mM per ml),suplemented with Penicillin and Streptomycin at 100 IU/ml and interleukin-2 (IL-2 500 IU/ml, Gentaur, Kampenhout, Belgium), medium further referred as the R10/IL-2 medium. PBMCs were cultured at a density of $2x10^6$ cells per ml at 37°C with 5% CO_2. R10/IL-2 medium was added on day 3 and 5 of culture. On day 7 cells were harvested, washed three

times with cold PBS (Gibco, Fisher Scientific, Waltham, Massachusetts, USA) and CD4+ T cells were isolated using negative selection magnetic beads (Miltenyi Biotech, San Diego, California, USA) according to the manufacturer's protocol. Purities of CD4+ T cells of more than 90% were confirmed by flow cytometry for CD3 (BUV496, clone OKT3), CD4 (BUV395, clone SK3) and CD8 (BUV737, clone SK1). Enriched cells were washed twice with R10/IL2 and then infected with HIV III_B lab strain (NIH AIDS reagent program) at a multiplicity of infection of 10^{-3} using spinoculation at 1200 g for 2 hours at 25°C followed by an additional 1 hour of incubation at 37°C 5% CO_2. Cells were washed twice with R10/IL2 and resuspended at $1x10^6$cells per ml in R10/IL2 medium to distribute for the VIA culture.

Preparation of CD8+ T effector cells: CD8+ T effector cells were prepared for two conditions : stimulated and non-stimulated. Freshly thawed PBMCs were stimulated with a potential T cell epitope (PTE) *gag* peptide pool (NIH reagent program) at 100 ng/ml per peptide in R10 medium and cultured for 7 days at $2x10^6$cells per/ml density without changing the medium. For unstimulated cells freshly thawed PBMCs were cultured overnight in R10 medium at $2x10^6$cells per/ml density. Both stimulated and unstimulated PBMCswere harvested on the same day and subjected to CD8+ T cell negative selection (Miltenyi Biotech, San Diego, California, USA). Purities of more than 95% ? 90% were confirmed by flow cytometry as described aboveCells were washed twice with R10/IL2 and resuspended at $1x10^6$cells per ml in R10/IL2 medium to distribute for the subsequent VIA culture.

The VIA was performed in flat bottom 96-well culture plate (Greiner BIO-ONE) in 200 µl volume of R10/IL2 medium. Each patient's sample was run in 5 conditions using triplicates: non-stimulated and stimulated CD8+ T effector cells in coculture with IIIb-infected CD4+ T target cells at an effector-to-target (E:T) ratio of 1:1 and an E:T ratio of 1:10, as well as IIIb-infected CD4 + T target cells only. Half of culture medium was recovered and refreshed every 3 days. Levels of p24 antigen in supernatants were quantified on day 14 in coculture by p24 ELISA (BioMARIC, Ghent, Belgium) according to the manufacturer's protocol. VSC was calculated as $\log_{10}$ [p24 CD4 + T cells only] – [p24 coculture with CD8 + T cells].

Measurement of soluble factors by MULTI-ARRAY ELISA

Several cytokines and chemokines were measured in plasma of patients and at day 5 of the VIA coculture using two different configurations of MULTI-ARRAY EKUSA mesoscale discovery (Rockville, Maryland, USA) U-plex detection plates respectively. On plasma we assesed IFN-γ, TNF-α, IL1-β, IL-6, IL18 and IP10. On supernatans from the VIA coculture we assesed IFN-γ, IL-2, IL6, IL-12p70, IP-10, TNF-

α, TRAIL, CCL4 (MIP1β). Technical procedures were done according to manufacturers instructions and read on the MESO SECTOR S600 reader.

IFN-γ ELISPOT

$MultiScreen_{HTS}$-IP, 0,45µm, 96 well plates (Merck Millipore, Burlington, Massachusetts, USA) were coated overnight with anti-human IFN-γ monoclonal antibody (clone 1-D1K, MABTECH, Stockholm, Sweden) diluted 1/100 in PBS at 4°C. The next day plates were washed with PBS and then blocked with R10 medium for 60 minutes. $2x10^5$PBMCs/per well in 200ul volume were loaded onto the plate supplemented with PTE *gag* peptide pool (NIH reagent program) at 100ng/ml concentration. Samples were run in two conditions: 7 days Gag-prestimulated PBMCs and non-stimulated PBMCs, each in duplicates for a total duration of 36 hours at 37°C 5% CO_2. Duplicate positive controls were stimulated with PMA (50ng/ml, Invivogen) and Ionomycin (500ng/ml, Invivogen). PBMCs from healthy donors were run as negative controls. After incubation plates were washed with PBS and incubated with biotinylated IFN-γ detection antibody (clone 7-B6-1, MABTECH) diluted 1/100 PBS-0,05 Tween-0,5%BSA for 120 minutes. Revelation was performed using HRP Streptavidin for ELISPOT (BD Biosciences) and AEC Substrate Set (BD Biosciences) according to manufacturer's instructions. Plates were read and analyzed analyzed using Easy-Count and the SmartCount (ImmunoSpot, Bonn, Germany) software.

Flow cytometry

Detailed cellular phenotyping of PBMCs using 18 colours flow cytometry was performed just before the isolation of CD8+ T cells for the VIA coculture and after 96 hours of coculture during the VIA. Before coculture, PBMCs were evaluated in parallel after 7 days of prestimulation with PTE *gag* peptides at 100 ng/ml per peptide or after overnight resting for unstimulated cells for a total of 16 hours with Golgi Stop (1/1000 final dilution, BD Biosciences). Golgi Plug (1/1000 final dilution) was added during the last 10 hours of culture together with an anti-CD107a antibody (BV421, clone H4A3).

During the VIA coculture, prestimulated CD8+ T cells with infected autologous CD4+ T cells or with non-infected autologous CD4 + T cells at the 1:1 E:T ratio were analyzed after 120 hours (5 days) in coculture. Golgi Stop (1/1000 final dilution, BD Biosciences), Golgi Plug (1/1000 final dilution) and anti-CD107a antibody (BV421, clone H4A3) were added into the culture 6 hours before staining.

Cells were further harvested for antibody staining, washed twice with 4 ml of FACS buffer (PBS with 1%BSA) and incubated with the LIVE/DEAD Fixable NearIR Dead cell stain kit (Thermo Fisher Scientific)and antibodies for the extracellular markers for 30 minutes at 4°C. Cells were washed twice

with 4 ml FACS buffer and permeabilized/fixed using the FOXP3 transcription factor staining buffer set (eBioscience, Santa Clara, California, USA) according to the manufacturer's instructions. Subsequently the intracellular markers were stained for 30 minutes. Cells were washed twice with 4 ml of FACS buffer and acquired on the Fortessa LSR flow cytometer (BD Biosciences, USA).

Different panels were used for phenoytping sharing a common backbone panel for lineage gating on T cells containing anti human antibodies for CD4 (BUV395, clone SK3, BD), CD3 (BUV496, clone UCHT1), CD8 (BUV737, clone SK1), CD45RA (BV460, clone HI100), CXCR5(BV711, clone J52D4), CCR7 (PE-CF594, clone 150305), CD14 (PECy5, clone G1D3) and LIVE/DEAD Fixable NearIR Dead cell stain kit (APC-Cy7, Thermo Fisher Scientific). The additional antibodies used in the panels were anti-human: HLA-DR (BV510, clone L243), IL2 (BV605, clone MQ1-17H12), CD57 (BV605, clone NK-1) IFN-γ (BV650, clone 4S.B3), PD-1 (BV786, clone EH12.1), Ki67 (Alexa Fluor 488, clone B56), CD38 (PERCP-Cy5, clone HIT2), TNF-α (APC, clone Mab11), IL-2 (PE, clone MQ1-17H12), Granzyme B (PE, clone GB11), Perforin (PE-Cy7, clone B-D48), MIP-1β (APC, clone D21-1351).

Quantification of HIV-1 DNA

Cell-associated HIV-1 DNA was isolated from purified CD4+ T cells (CD4+ T cell microbeads, Miltenyi) at final quantities of $2x10^6$ cells for DNA per patient. Extraction was done using the DNeasy Blood and tissue kit (Qiagen, Hilden, Germany) according to manufacturer's instruction. Absolute quantification of HIV-1 DNA was performed by droplet digital PCR (ddPCR) (Bio-Rad, Hercules, California) as previously described (35) with total HIV-1 DNA primers and probes used as reported (36).

Data analysis and statistics

Flow cytometry data were analyzed using Flow Jo (version 10, BD Biosciences) and Kaluza (version 1.5, Beckman Coulter). Unsupervised ViSNE analysis was executed in the cytobank environment (Beckman Coulter). Briefly cleaned files of CD3+ T cells were uploaded and clustering was executed based on lineage (CD4,CD8), prolferation (Ki67) and functional markers (IFN-γ, CD107a, Perforin, Granzyme B). Manual analysis was done by a classical gating strategy focused hierarchically on lymphocytes,single cells, live-cells, CD3+,CD8+ cells. Subsequently CD8+ T cells were subdivided into the four subsets (Naïve (CD45RA+CCR7+), Central Memory (CM) (CD45RA-CCR7+), Effector memory (EM) (CD45RA-CCR7-) and terminal effector memory subsets reexpressing CD45RA (TEMRA) (CD45RA+CCR7-). Each subset was analysed with a Boolean gating strategy combining 8 markers. Data was exported as .csv files and imported into the Tableau (Tableau Sotware, Seattle, Washington, USA) environment for data cleaning and organization. Statistical testing was executed with the QluCore (Qlucore, Scheelvägen,

Sweden) software using Kruskal Wallis non-parametric comparison with a false discovery rate (FDR) of 0.2. Identified subsets were exported into Prism version 8 (GraphPad Software, California, USA) and represented with levels of significance from non-parametric multiple comparison with Dunn's correction.

All other data was analysed with Prism (version 8, GraphPad) and comparison were done using non-parametric statistics (specific tests indicated below each graph)

5.4 RESULTS

Participants demographics, plasma markers and cell associated HIV-1 DNA

The characteristics of HIV-1 patients recruited in the PhenoCure cohort are given in Table 1. Inclusion criteria are mentioned in the material and method section defining the three categories. Importanly EC and VC were treatment naïve with the exception of two EC women which have been ART-treated only shortly during one pregnancy. Information on the specific subtype of HIV-1 was not available for all patients.

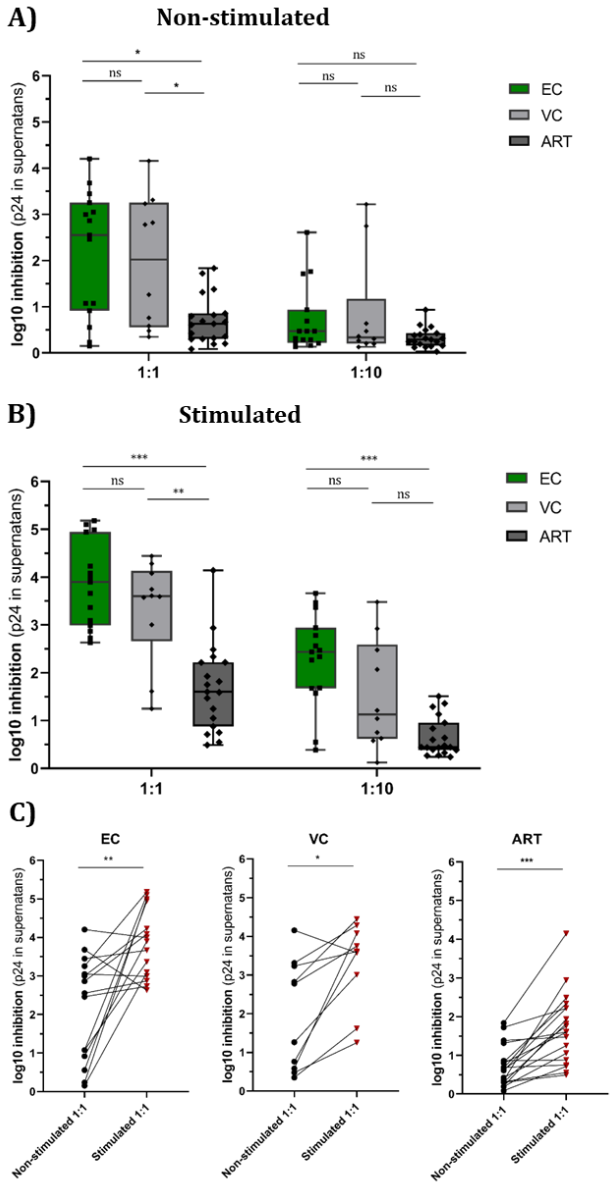
Category	All	EC	VC	ART
Number of individuals	56	15	11	30
Median **Age** (years)	48	50	44	49
Range	27-69	34-69	27-65	28-68
Median **CD4 counts** (cells/µL)	863	937	936	778
Range	542-1980	712-1450	566-1980	542-1487
Median **CD4 nadir** (cells/µL)	552	665	647	299
Range	63 - 848	231-848	340-748	63-801
Median **current pVL** (copies/ml)	< 20	< 20	86	< 20
Range	19-1280	/	19-1280	/
Median **highest pVL recorded** (copies/ml)	16800	< 20	182	469750
Range	19-10000000	19-11400	54 - 47800	5470 - 10000000
Median **HIV-1 DNA** (copies/million CD4)	1013	124	185	3680
Range	46 - 11146	46 - 285	68 - 9648	208 - 11146
Sex (percentage of male participants)	74%	40%	45%	80%

Table 1. Patient characteristics recruited in the PhenoCure cohort (pVL: plasma viral load)

With regard to plasma markers an elevated concentration of circulating IL-18 in ART patients (median 534,8 pg/ml, [290,9 – 1478]) compared to EC (336 pg/ml, [149,1 – 412,1]) was observed (supplementary figure 1, A), suggesting that systemic inflammation stays elevated during ART and in contrast is more limited during natural control of HIV-1 infection. All other tested cytokines did not differ among patient groups (supplementary figure 1, B-D).

Cell-associated total HIV-1 DNA was significantly lower for EC (median 124 copies/million CD4 + T cells, [46 – 2530]) compared to the ART group (median 3680 copies/million CD4 + T cells, [208 – 11146]) whereas the values for VC (median 185 copies/million CD4 + T cells, [68-9648]) were not significantly different between EC and ART-treated patient (supplementary figure 2, A). These findings are concordant to published literature (37).

Viral suppressive capacity significantly increases by Gag stimulation and is more pronounced during natural control of HIV-1 infection



Increased VSC is commonly observed during *in vivo* viral control. In the present study, two first questions were addressed: First, to what extent will prestimulation with Gag peptides improve VSC of CD8 + T cells compared to non-stimulated, baseline VSC. Second, how this potential induction of VSC compares among different clinical phenotypes, namely HIV controllers (EC and VC) and treated progressors (ART). In addition we ran VIA assays using two E:T ratios (1:1 and 1:10) to estimate the per cell capacity of suppression. In the non-stimulated setting VSC in 1:1 ratios is significantly increased for EC and VC as compared to ART (median VSC in log_{10}: EC 2.5, VC 2.02, ART 0.63). When effector cell to target cell ratio was reduced (1:10), VSC decreased so that no further difference among groups was observed (figure 1, A) (median VSC in log_{10}: EC 0.47, VC 0.33, ART 0.29). After Gag PTE pre-stimulation for 7 days, VSC in EC and VC was still significantly higher than in ART patients (median VSC: EC 3.9, VC 3.6, ART 1.6.).

Figure 1. Viral Suppressive capacity is highest in EC and significantly increased after peptide stimulation for all patient groups. Log_{10} inhibition of HIV p24 production measured for non-stimulated (A) and Gag PTE peptide pool (NIH) stimulated (B) CD8+ T cells towards superinfected autologous CD4+ T cells at E/T of 1:1 and 1:10 (sample size, EC (n=15), VC (n=10), ART (n=18)). Statistical testing by non-parametric Kruskal Wallis with Dunn's correction for multiple comparison. VSC compared between non-stimulated and stimulated conditions for each patient group (C) tested by Mann Whitney non-parametric statistics.

Interestingly VSC stayed significantly elevated for EC when ten times less effector cells were present (figure 1, B) (median VSC: EC 2.4, VC 1.1, ART 0.4). This points to a higher presence of HIV-1 specific T

cells or an increased per cell based ability to suppress viral outgrowth. Interestingly stimulation with the Gag peptides enhanced VSC in all patient groups (figure 1, C). The ratio of enhancement for VSC stimulated over non-stimulated condition (EC. 1.56, VC 1.78, ART 2.54) was the highest for ART patients.

Colletively, these data underline the importance of anti-Gag CD8+ T cell response for viral suppression across different clinical phenotypes. EC and VC harbored a high VSC already at baseline, which further increased upon stimulation, ART patients, profit significantly from prestimulation as well.

IFN-γ secretion increased after stimulation and correlates moderately with VSC

IFN-γ secretion, measured by ELISPOT, is a hallmark of antiviral immunity and is frequently used to assess the effect of T cell vaccination strategies in clinical trials.

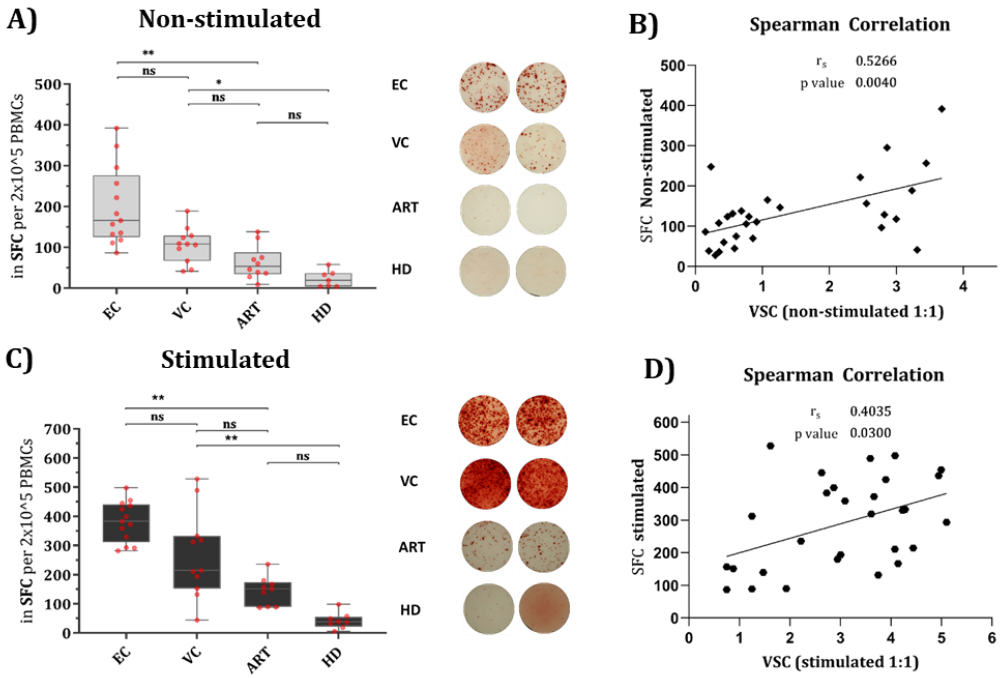


Figure 2. IFN-γ secretion is significantly increased in EC and correlates moderately with VSC
Overnight rested PBMCs were either not pre-stimulated (A) or pre-stimulated with Gag PTE peptides for 7 days (C) before IFN-γ secretion of PBMCs was measured by ELISPOT during 36 hours in the presence of Gag peptides. Results are depicted as spot forming counts (SFC) and representative duplicates. (sample size, EC (n=13), VC (n=10), ART (n=10)) Statistical comparison by Kruskal Wallis non-parametric statistics with correction for multiple comparison by Dunn's method. All HIV-1 infected patients were analyzed for correlation with VSC by Spearman's rank correlation for non-stimulated (B) and pre-stimulated (D) PBMCs.

In order to know whether secretion of IFN-γ does correlate with VSC we assessed the number of spot-forming cells (SFC) in non-stimulated PBMCs and Gag PTE-stimulated PBMCs. In both conditions, HD PBMCs showed minimal responses, as expected; ART patients showed a weak response as well (not significantly different from HD), but enhanced by stimulation (figure 2, A). In EC the response was heterogenous but significantly increased from VC and ART when PBMCs were not-stimulated (figure 2, A). After stimulation for 7 days ELISPOT responses increased in all patient groups as compared to their respective non-stimulated condition (Wilcoxon paired rank test, EC: $p < 0.0005$, VC: $p < 0.005$ and ART: $p< 0.005$). The fold change of stimulated versus non-stimulated (ratio of median values) condition per patient group was: EC (2.25), VC (1,98), ART (2.84). Hence, the number of IFN-γ secreting cells increased most in ART patients. Of note EC had still the significant highest total measures after peptide

stimulation for 7 days (median SFC, EC: 392, VC: 214, ART: 151). Overall, the number of IFN-γ secreting cells does correlate moderately with VSC for both conditions (figure 2, B and D).

Upon Gag peptide pre-stimulation CD8+ T cells increased Ki67 but IFN-γ and CD107a are significantly upregulated in HIV controllers only

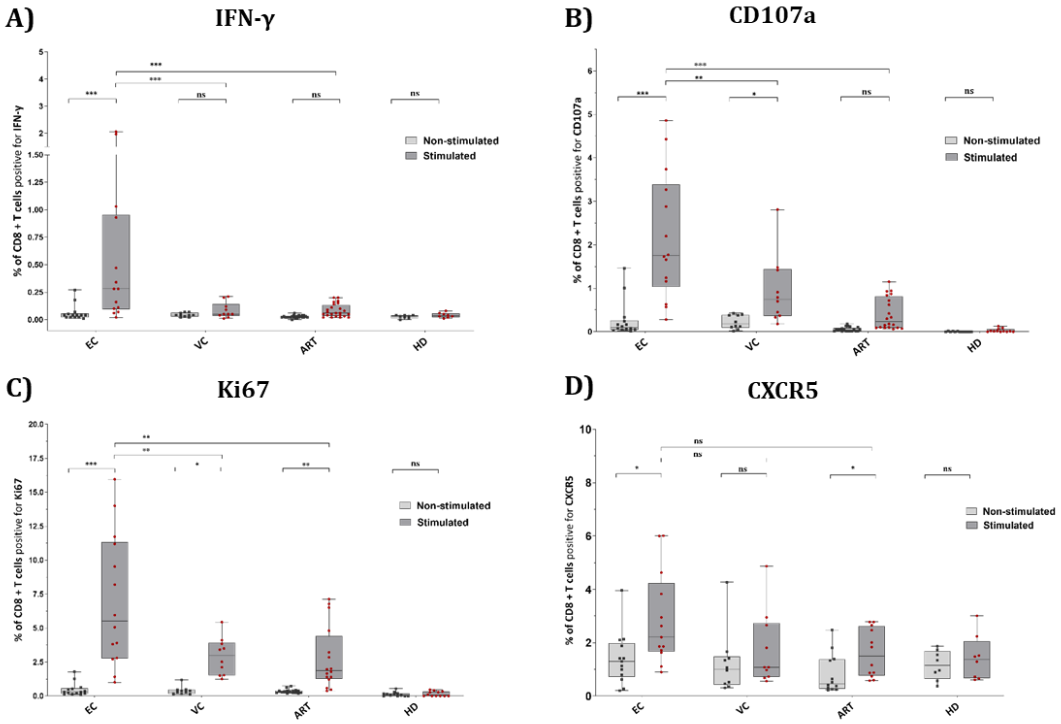


Figure 3. CD8 + T cells of EC express significantly more IFN-γ, CD107a, Ki67 and CXCR5 after peptide stimulation
Expression of IFN-γ (A), CD107a (B), Ki67 (C) and CXCR% (D) on CD8 + T cells non-stimulated (light grey) and stimulated (dark grey) (sample size, EC (n=15), VC (n=10), ART (n=18)). Statistical comparison by Kruskal Wallis with Dunn's correction for multiple comparison across groups and a paired Mann Whitney Wilcoxon rank test for each non-stimulated versus stimulated within group comparison.

In those conditions, EC show significantly increased production of IFN-γ in CD8+ T cells after Gag PTE prestimulation compared with non-stimulated PBMCs but also compared to other patient groups (figure 3, A). Remarkably, pre-stimulation selectively enhances IFN-γ production in EC and not in VC or ART (figure 3, A). CD107a expression is similarly low in non-stimulated cells from all groups and is significantly increased EC and VC after pre-stimulation, but much more so in EC than in VC or ART patients (figure 3, B). A similar trend is observed for Ki67, a proliferation-associated marker, but the increase in expression after pre-stimulation, is significant in all HIV (+) subjects, including ART. However, EC show still significantly higher percentages of Ki67 positive cells than VC and ART (figure 3, C). Interestingly an increase in CXCR5 is oberved on CD8 + T cells, which was significant for EC and ART (figure 3, D). The analysis of bulk CD8 + T cells showed no difference in Granzyme B or Perforin (data not shown). There was no correlation with VSC for IFN-γ, CD107a and CXCR5. Ki67 correlated

only weakly with VSC (supplementary figure 3). Overall, these data showed that all HIV-1 patients respond to Gag stimulation by upregulation of the proliferation marker, Ki67. IFN-γ and CD107a however were selective attributes of natural control.

EC develop distinct and multifunctional cytotoxic CD8 + T cell subsets upon stimulation with potential access to lymph node follicles

To further assess the phenotypical characteristics of stimulated CD8 + T cells we analyzed flow cytometry data by unsupervised ViSNE clustering for combinatorial expression of functional markers in memory and effector subsets (supplementary figure 7). According to these analyses, we found several rather unique multifunctional subsets in EC (figure 4) as shown in a 2D plot (figure 5). Thus two distinct subsets with a cytotoxic profile expressing Ki67, IFN-γ, CD107a, Perforin and Granzyme B were observed, in the effector memory (figure 4, A) and the central memory compartment (figure 4, B). Both subsets were nearly absent in VC or ART patients and correlate well with VSC over the whole cohort (figure 6). Interestingly a third distinct subset with expression of CXCR5 and several cytotoxic markers was identified (figure 4 C) which was significantly more prevalent in EC compared to ART and correlate well with VSC (figure 6). In summary, the identified subsets suggest the importance of tissue homing such as potential access to the germinal centers, the B cell zone of lymph nodes.

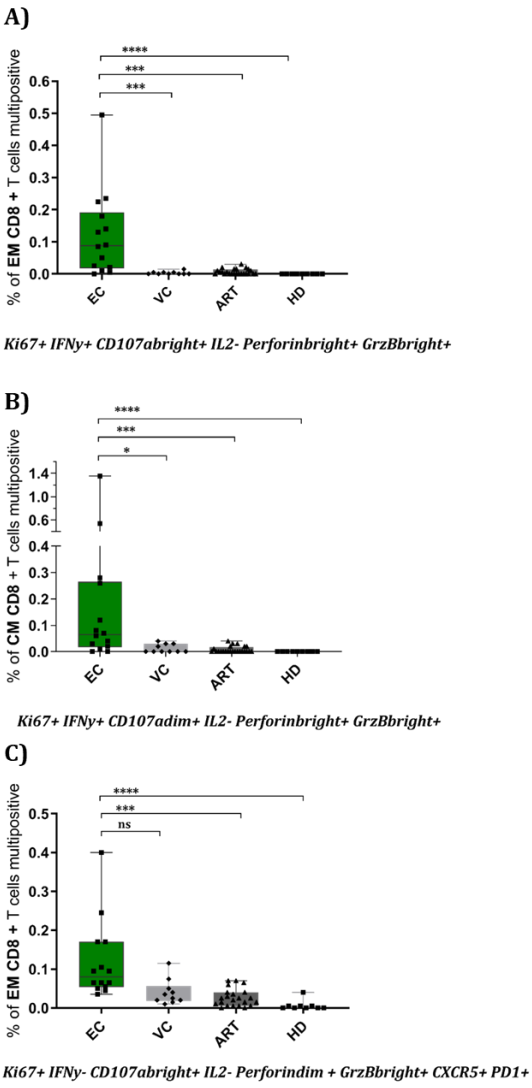


Figure 4. CD8 + T cells of EC show multifunctional, cytotoxic profiles in effector and central memory compartment
Phenoytpes of CD8 + T cells were assesed upon stimulation for 7 days by flowcytometry. Combinatorial expression of different markers was analyzed by boolean gating strategy. CD8+ effector memory T cells (CCR7-CD45RA-) in (A, C) and central memory T cells in (B). The specific expression pattern is annoted below the graphs. (sample size, EC (n=13), VC (n=10), ART (n=12)) Statistical testing in two steps, first the identification of hits by Kruskal Wallis with a FDR of 0.2 followed by non-parametric Kruskal Wallis comparison with Dunn correction for multiple comparison.

Interestingly a third distinct subset with expression of CXCR5 and several cytotoxic markers was identified (figure 4 C) which was significantly more prevalent in EC compared to ART. In summary, the identified subsets suggest the importance of tissue homing such as potential access to the germinal centers of B cells in lymphoid tissues.

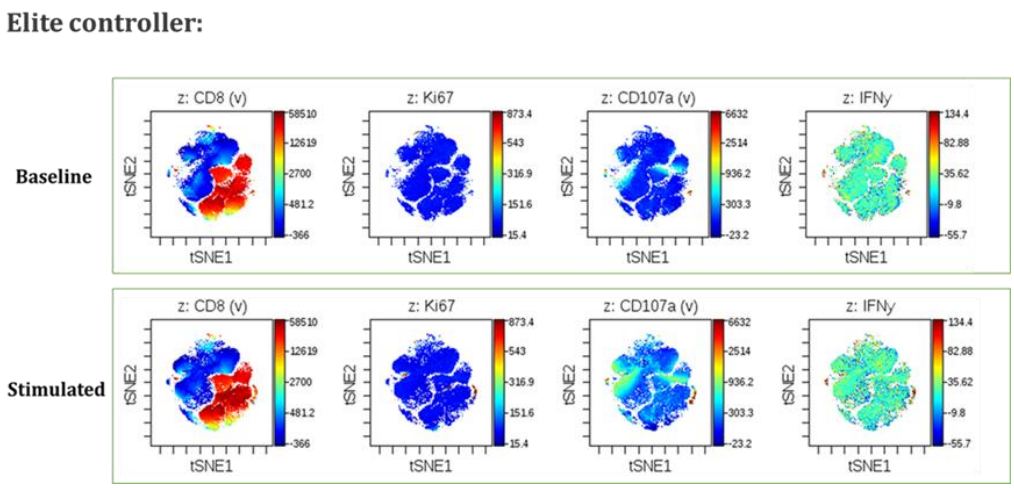


Figure 5. Unsupervised ViSNE analysis reveals multifunctional subsets in EC expressing Ki67 CD107a and INFγ. 2D clustering by ViSNE depicts 13 dimension of CD8 + T cells as shown in this exemple of a single EC file

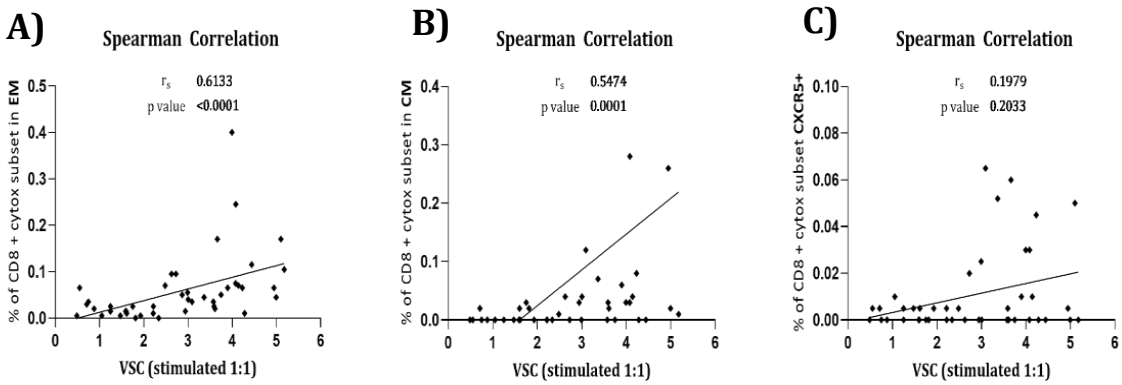


Figure 6: Multifunctional subsets of EM and CM compartment correlate well with VSC
Multifunctional subsets identified by boolean gating of PBMCs stimulated for 7 days (figure 4) and the corresponding correlation with VSC for EM (A), CM (B) and the subsets with CXCR5 expression (C). (sample size, EC (n=15), VC (n=10), ART (n=18)). Spearman correlation with statistical values plotted on graph.

No differences in cellular phenotype nor soluble factors during the coculture of VIA

To unravell the mechanism of action of CD8 + T cells during VIA we performed flow cytometry at 96 hours in coculture (Figure 7). Strong expression of the proliferation marker (Ki67), the effector molecule (Perforin) and terminal differentiation marker (CD57) was found in all patient groups. Especially the high levels of Perforin suggested an involvement of direct cell to cell killing. However, single marker expression levels did not show any significant differences between patient groups. Boolean analysis of combinatorial expression revealed no significant differences among clinical phenotypes as well (data not shown).

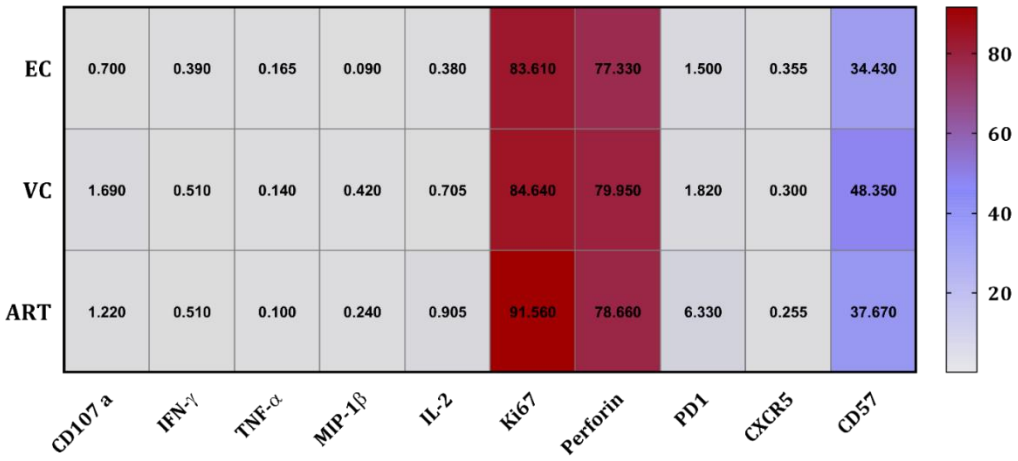


Figure 7. No significant difference in expression of various proteins on CD8 + T cells on Day 5 in coculture
Heat Map depicts median expression of proteins in columns per patient group in rows. Median values are plotted inside of heat map cells. (sample size, EC (n=15), VC (n=10), ART (n=12)) Statistical testing by non-parametric statistics revealed no significant differences among patient groups.

Although only exploratively, we observed in seven patients a trending difference in CD8+ T cells coexpressing Ki67, Perforin and IFN-γ at only 48 hours in coculture (Supplementary figure 5). No other significant differences were revealed in other soluble markers (IFN-γ, IL-6, IP10, MIP-1β, TNF-α and TRAIL) from the culture supernatants at day 72 hours in coculture (supplementary figure 6).

5.5 DISCUSSION

Evidence for the importance of anti HIV-1 CD8 + T cell responses during natural control continues to substantiate. In our study, we evaluated two aspects of *in vitro* viral suppression in patients with natural control of HIV-1 infection and well-treated progressors: the *ex vivo* "effector function" (unstimulated condition) as well as the "memory function" (after *in vitro* stimulation with Gag peptides). We focused specifically on the immune phenotypes of CD8 + T cells and their functionality. While VSC did not correlate with the viral reservoir or the clinical parameters of patients, we observed a clear association with IFN-γ secretion and proliferation markers of CD8 + T cells. Furthermore, we identified cytotoxic subsets with tissue homing abilities specific for natural control of HIV-1 and correlated with VSC. One of these Gag reactive memory subsets express CXCR5 and might have potential access to the B cell zone of lymphoid tissues. Overall, we show that the use of a polyvalent Gag peptide pool induces a significant increase of VSC in HIV-1 infected patients, including treated progressors, supporting the implementation of Gag-directed immunotherapy towards functional cure.

Several studies have reported elevated *ex vivo* VSC in EC compared to progressors providing a direct link between control of viremia and anti-viral CD8 + T cell immunity (9,25,26,28,38,39). We observed a significant higher suppression of EC in non-stimulated CD8 + T cells as well, however with a higher heterogeneity across EC compared to other reports (figure 1 A). Interestingly the use of lower E:T ratio (1:10) abrogates suppression of non-stimulated CD8 + T cells in our setting in contrast to what Cirion et al. have reported. They observed the same level of VSC for HIV controllers in 1:1 and 1:10 ratios (38). Possible reasons for this divergent findings could be slightly different technical setup for the VIA and prestimulation of CD4 + Tcells using different gag peptide pools. In addition, could the use of cryopreserved PBMCs instead of freshly isolated PBMCs be decisive. Aware of this caveat we applied the established procedure of overnight resting for functional studies on lymphocytes before use to limit interference (40,41). Still we cannot totally exclude an effect of cryopreservation.

In contrast with most other studies, we included VC and EC as two distinctive groups. First and foremost, it has to be emphasized that the current median pVL in this group was 86 copies/ml (table 1). Consequently, at this low range a big proportion of these patients were categorized as EC in the past when detection limits for pVL were at 50 copies/ml or higher. Therefore, caution is advised when relating to the existing literature. Secondly, most studies either do merge EC and VC or just look at EC. Consequently, studies on VC are comparably scarce. Our data shows that VC exert comparable VSC to EC (figure 1 A). The study from Spentzou et al. has reported similar results (34). This suggests that slightly elevated pVLs do not reflect a loss of CD8 + T cell suppression. Interestingly at the current

understanding elevated pVLs could even contribute to the maintenance of anti-viral CD8 + T cell responses as resolving viremia during the start of ART associates with loss of VSC in progressors (39). We did not find any correlation between pVL and VSC (supplementary figure 4) and confirmed that VSC associates with natural control of HIV-1.

In several previous studies Gag-specific CD8 + T cell responses (measured by ELISPOT or flow cytometry) were inversely correlated to viral load and were also described to be a major contributor to increased VSC (27,28,42). We sought to investigate the potential of 7-day Gag stimulation using the PTE Gag peptide pool (NIH reagents programm) across the different patient groups. These peptides cover multiple overlapping sequences reflecting the natural diversity of circulating HIV-1 strains (21). Consequently, they offer a large antigenic breadth and depth which refers to coverage of potential T cell epitopes and cross recognition of variants of these epitopes respectively. Hence many are clustered in conserved regions (43–45). Likewise, the importance of breadth in the anti-Gag response during control of HIV-1 has been underscored (46,47). To this end, we hypothesized that expanding such memory T cells has most clinical significance as it allows to boost the VSC of ART patients and at the same time delineate relevant T cell subsets of progressors from controllers potentially implicated during clinical non-progression.

We observed that VSC increased significantly after stimulation with PTE Gag peptides in all patient groups with several orders of magnitude (figure 1, C). This reflects the importance of anti-Gag responses possibly rooted in their rapid recognition as Gag can be presented and recognized on HLA-I molecules even before viral integration (48,49). The ability to redirect CTL killing against reactivated autologous virus by Gag stimulation was reported previously for EC and ART, however using IL2 during prestimulation of CD8 + T cells (50). Notably both controller groups showed significantly higher VSC after peptide stimulation as compared to ART (figure 1, B). This might be rooted in an increased immunodominance of anti-Gag T cells of controllers (51,52), which could be further amplified by their expansion. The significantly higher expression of Ki67, a proliferation marker, on CD8 + T cells of EC could support this hypothesis (figure 3, C). Importantly the proliferation of antigen specific CD8 + T cells was associated to natural control by other studies as well (10,11). Shan et al. also observed increased proliferation for ART patients in response to Gag peptides (50), our data showing significant higher expression of Ki67 pointing into a similar direction (figure 3, C). Next, we see that the VSC of EC after pre-stimulation with Gag PTE stays elevated even when E:T ratio is decreased to 1:10 (figure 1, B) indicating a higher magnitude in Gag specific CD8 + T cell expansion (as shown by higher Ki67 expression in EC) (figure 3, C) or a stronger per cell suppressive capacity.

To explore this aspect in detail we applied IFN-γ ELISPOT at baseline and after seven-day peptide stimulation. ELISPOT results clearly showed the increased frequency of IFN-γ-secreting T cells in PBMCs of EC at baseline and after pre-stimulation (figure 2, A and C). We observed only moderate correlation with VSC in both settings (figure 2, B and D). Other studies reported also divergent results (28,53). Interestingly an *in vitro* vaccination study reports a correlation with IFN-γ only when coexpressed with CD107a and MIP-1β (54). This suggests that not IFN-γ alone but rather multifunctionality might be the best reflector of increased VSC.

To explore polyfunctionality on a single cell level we phenotyped by eigtheen color flow cytometry before and after seven days of peptide stimulation. The expression of IFN-γ in total CD8 + T cells was significantly increased only after stimulation between EC and VC and ART (figure 3, A). The degranulation marker CD107a increased significantly for both groups, VC and EC (figure 3, B). Interestingly Ki67 expression increased significantly in all three groups which was suggestive of a specific HIV-1 proliferative response to the Gag epitopes (figure 3, C), and corroborated by its strict absence in HD controls (figure 3 A-C), but was finally shown as a moderate correlate of viral suppression *in vitro*.

Polyfunctional anti-viral CD8 + T cells are a qualitative feature of EC (18,45,55). To assess if the combinatorial expression of functional markers on Gag stimulated CD8 + T cells delineates natural control and progressors we first applied unsupervised ViSNE analysis (exempligied in figure 5). It allowed us to define the most distinct subsets on a first and unsupervised view. We tracked back those subsets by boolean gating strategy of our raw data. This way we identified three distinct subsets significantly increased in EC. Two of those showed strong cytotoxic characteristics (figure 4, A-B) of EM and CM compartment, which correlated well with VSC (figure 6, A-B). This provides evidence for the co-existence of multiple subsets that govern HIV-1 control in distinct memory CD8 + T cells in central lymphoid organs as well as in peripheral tissues.

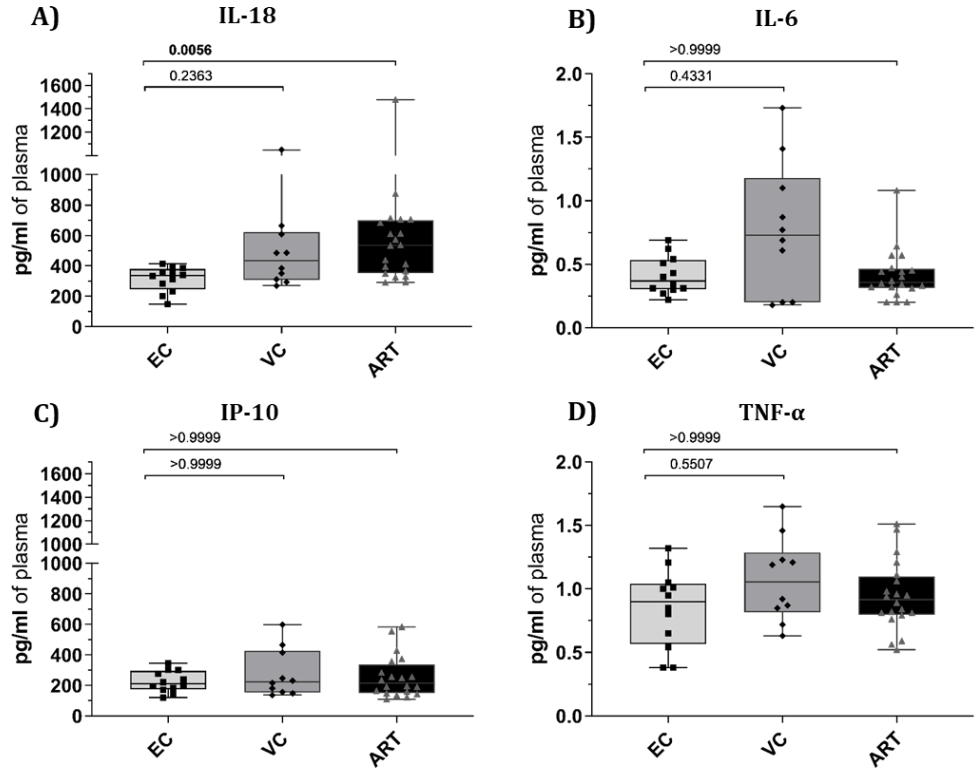
Intriguingly Gag peptide stimulation upregulated CXCR5 in EC and ART patients (figure 3, D). Recent studies suggest that circulating CXCR5 + CD8 + T cells are major producers of IL21 and associate with limited HIV-1 replication (56). We cannot confirm differences either at baseline or after peptide stimulation. However a cytotoxic subset expressing CXCR5 with potential access to the B cell follicles (figure 4, C) a major site of the viral reservoir (57,58) was significantly expanded in natural controllers. Recent literature is controversial on the functionality of these cells. Reuter et al. have reported a strong correlation between CXCR5 + CD8 + T cells isolated from lymph node (LN) and pVL in VC but a

decreased killing ability compared to bulk CD8 + T cells from PBMCs of the same patients(59). In contrast, although Petrovas et al. observed a similar strong correlation with pVL they find increased killing capacities of follicular CD8 + T cells from LN as compared to CXCR5 negative counterparts despite their compromised cytokine profile (60). To our knowledge, we characterized for the first time a polyfunctional CD8 + T cell subset combining features of cytotoxicity, proliferation and expressing CXCR5 after expansion with Gag peptides. The only weak positive correlation with VSC (figure 6, C) could eventually be rooted in the low presence of this subset in the blood. A thorough analysis of their cytotoxic capacity demands a single cell sorting. Clinically these cells might be of utmost importance since follicular helper CD4 T cells (TFH), residing in B-cell follicles of secondary lymphoid tissues represent a major site of viral persistence (57). Future studies need to confirm that the CXCR5 positive subsets have suppressive abilities and have access to the LN of HIV controllers.

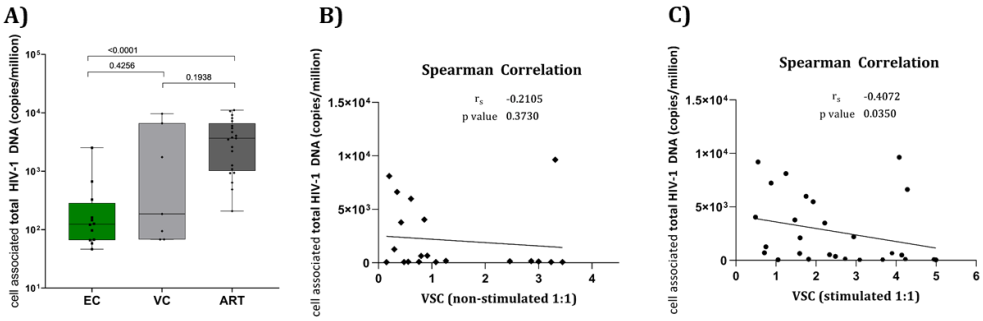
To elucidate the mechanism of HIV-1 CD8 + T cell mediated suppression *in vitro* during the VIA coculture we phenotyped T cells 96 hours after the coculture when solid viral replication was observed by intracellular P24 staining (data not shown). Although we found no significant differences between patient groups, the strong increase of Perforin on total CD8 + T cells of all patients (figure 7) suggests a direct cytotoxic mechanism as previously proposed (9). Interestingly we detected perforin, IFN-γ and Ki67 positive subsets at 48 hours between EC and ART (supplementary figure 5) in a subset of seven patients suggesting that the assesment for phenotypic differences should be probably done earlier, before notable viral replication. At 96 hours in coculture the effect of a high concentration of exogenous IL-2 supplemented to the VIA culture might have already levelled out phenotypic differences between clinical phenotypes and lead to similar levels of cytokines or chemokines at 72 hours in coculture (supplementary figure 6) Overall the exact mechanism of viral suppression *in vitro* remains to be determined.

In conclusion, our work demonstrated that stimulation with polyvalent Gag peptide pools efficiently increased VSC in all group of patients but with a greater extend in EC. Interestingly upregulation of CXCR5 expression was noted. The increased frequencies of several distinct multifunctional CD8 + T cells subsets, including one subset expressing CXCR5, is an attribute of elite control. CXCR5 + CD8 + T cells represent a unique subset of CD8 + T cells that may play a significant role in the containment of reservoirs. Overall, our data highlights a multi-qualitative anti-Gag CD8 + T cell response during control of HIV-1 that should be elicited for future vaccination strategies to achieve viral remission.

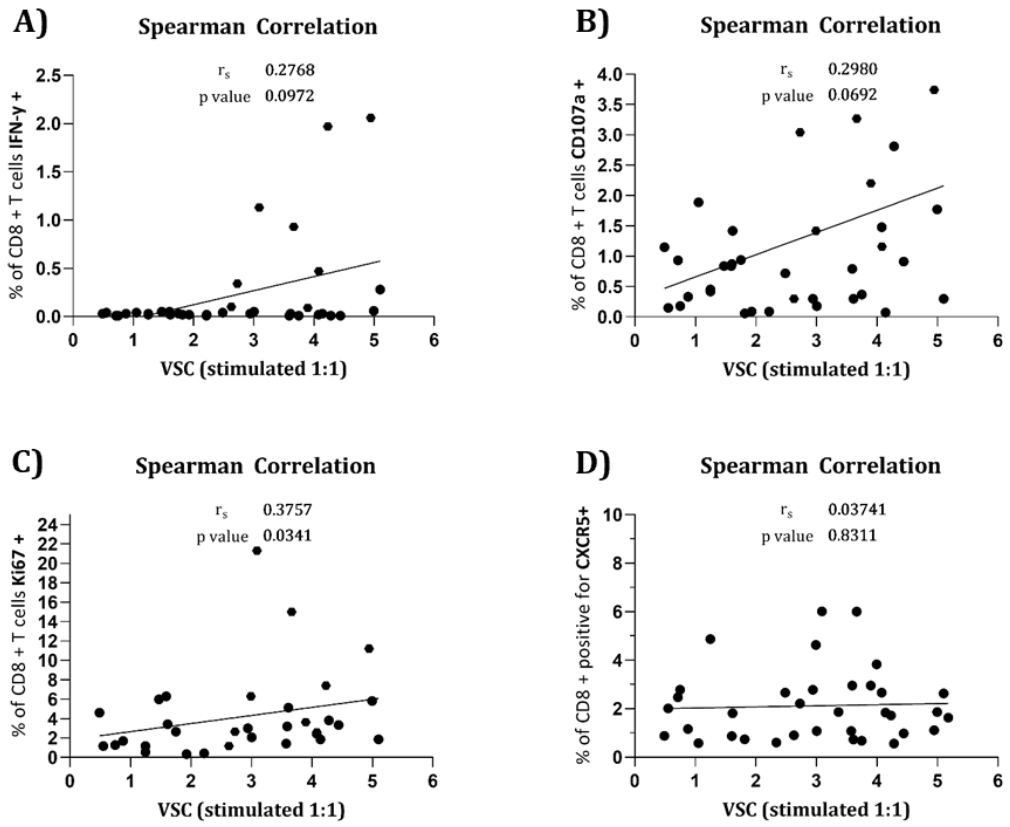
5.6 SUPPLEMENTARY MATERIALS



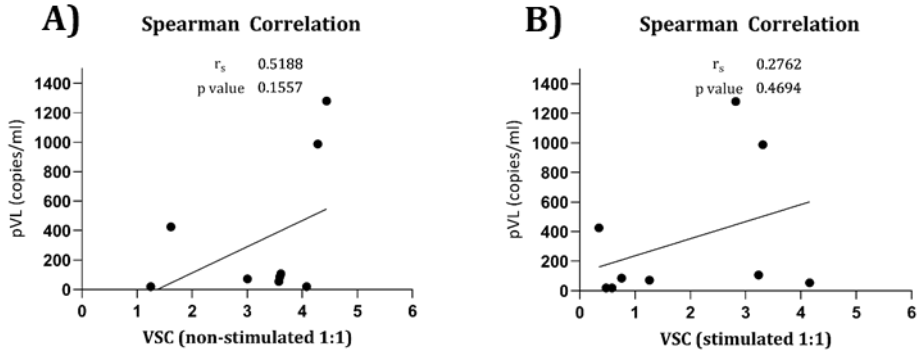
Supplementary figure 1. IL18 concentration in plasma differs significantly between EC and ART
Plasma samples isolated during blood donation were analysed for the concentration of cytokines IL18 (A), IL-6 (B), IP-10 (C) and TNF-α (D). Statistical testing by non parametric Kruskal Wallis test with Dunn correction for multiple comparison.



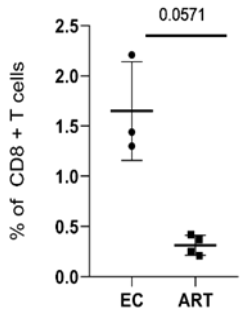
Supplementary figure 2. HIV-1 DNA is significantly lower in EC compared to ART and has a weak negative correlation with VSC
Absolute quantification of cell associated HIV-1 DNA on purified CD4 + T cells by ddPCR.Five samples (A) (two of EC and three of VC measurements were below the confidence interval of the negative controland cannot be accurately quantified. These values have been excluded from the analysis to precludestatistical bias). Testing was done with Kruskal Wallis non-parametric statistics with Dunn correction. Spearman correlation of cell associated HIV-1 DNA with VSC on non-stimulated (B) and stimulated (C) cells.



Supplementary figure 3. No correlation with IFN-γ, CD107a and only weak correlation with Ki67 expression and VSC
Correlation of CD8 + T cell expression of IFN-γ (A), CD107a (B) and Ki67 (C) with VSC. Non-parametric rank spearman correlation with rho and p values given on the graph.

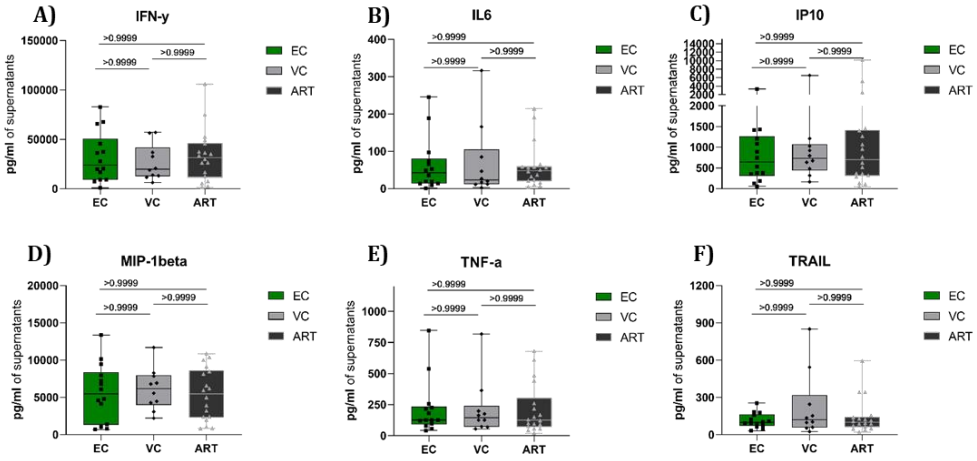


Supplementary figure 4. No correlation of pVL with VSC of VC
Correlation of pVL with VSC non-stimulated (A) and with stimulated (B) CD8 + T cells given for VC only because all other patients of the cohort have undetectable viremia. Statistical values plotted in the graph.



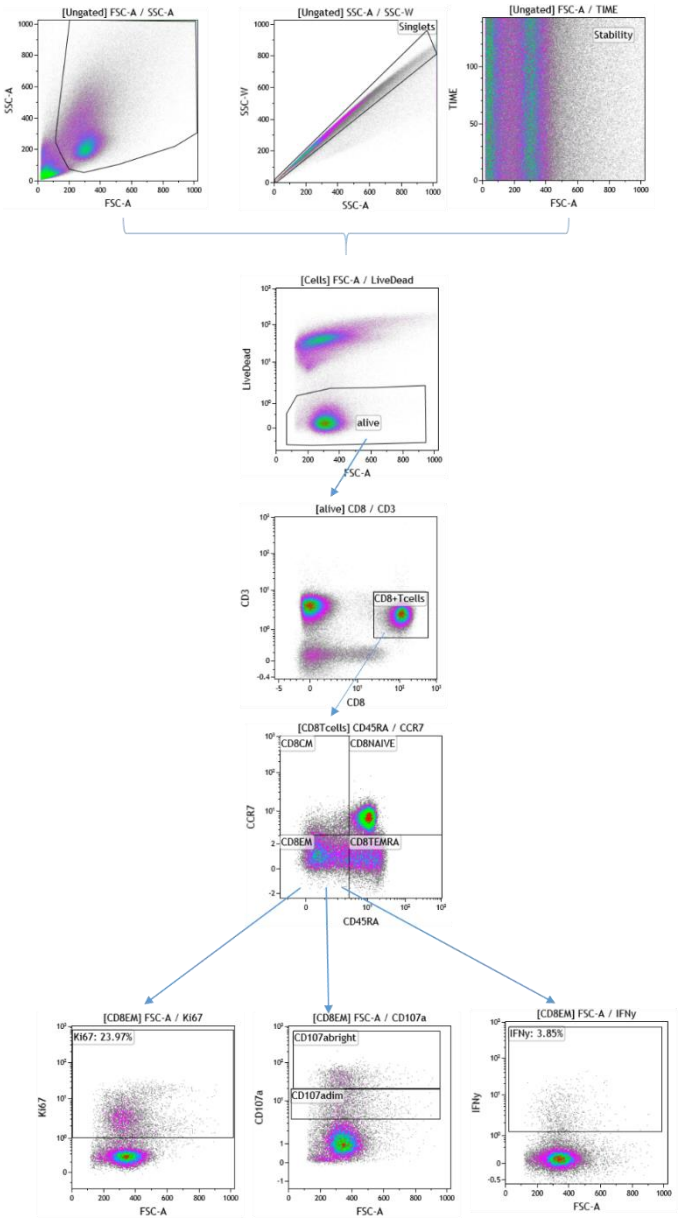
Supplementary Figure 5. Slight trending difference between EC and ART patients of Ki67, Perforin and IFN-γ co-expressing CD8 + T cells at 48 hours in coculture

To explore the kinetic pattern of cytotoxic markers during coculture we phenotyped 7 patients at 48hours.Statistical testing by Mann-Whitney non-parametric comparison.



Supplementary figure 6. Concentration of cytokines at 3 days in VIA coculture reveal no differences between patients

Supernatants of coculture at 72 hours (day 3) were tested with mutliplex ELISA for IFN-γ (A), IL-6 (B), IP-10 (C), MIP-1β (D), TNF-α (E) and TRAIL (F). Statistical testing by non-parametric Kruskal Wallis test with Dunn correction for multiple comparison



Supplementary Figure 7. Hierarchical gating strategy of flow cytometry data

The basic gating strategy starts with the combined parameters of size and granularity (FSC, SSC), singlets (SSC-W, SSC-A) and stability (time, FSC-A) defined as «cells» gate. Next the dead cells are excluded followed by identification of CD3+CD8+ events as «CD8Tcells». Then we can distinguish the four main memory population based on CCR7 and CD45RA expression. In the last step all remaining markers are plotted against FSC-A (here exemplified for effector memory subsets and Ki67, CD107a and IFN-γ) (gates are defined based on FMO controls). Based on those gates boolean combination of markers was be applied to identify small CD8 + T cell subsets with mutiple features.

5.7 REFERENCES SECTION 5

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6 GENERAL DISCUSSION AND PERSPECTIVES

Within less than two decades, HIV-1 infection has turned from an automatic death sentence into a treatable chronic infection (1,2). In fact, current drug regimens allow patients a nearly normal life span and even abort the risk of unprotected sexual transmission (3,4). Clearly, the major limitation of ART is that it is not curative as HIV-1 persists in treatment refractory viral reservoirs (chapter 2.9). Therefore, life-long therapeutic adherence is mandatory and leads to new challenges. Nevertheless, before discussing the current status of global ART based healthcare and the HIV/AIDS pandemic a contextualization of the presented research data is needed.

In 2009 the first case of HIV cure was reported and did considerably boost the interest into the field of cure research. Timothy Brown, the Berlin patient, is the living proof that it is possible. He underwent an extensive immune ablation regimen in combination with transplantation of hematopoietic stem cells harboring the homozygous CCR5Δ32 genotype and remains undetectable till this very day (5). Although attempted multiple times, today ten years later only one recent report suggests a similar achievement, in London this time (6). More importantly, the life threatening procedure of immune ablation and allotransplantation clearly limit its use to a small group of cases only. Therefore, alternative strategies to approach a cure are pursued. That said it became clear over the last years that a full eradication of viral reservoirs from the body, referred to a sterilizing cure, is very difficult to achieve. Mainly because the purging of latent virus by latency reversal agents or LRA is insufficient or toxic (7). The concept of a functional cure in contrast refers to control over HIV-1 in the absence of drug therapy although the cell-associated virus DNA remains detectable. Several clinical studies have underlined that rebound of viremia from patients is far less likely upon treatment interruption when reservoirs are sufficiently reduced in size (8,9). Next to that a more functional anti-viral immunity is a common observation during spontaneous long-term non-progression of HIV-1 (10). Taken together the current consensus defines a reduced viral reservoir in size combined with improved anti-viral immunity as preferable factors to achieve a functional cure.

When this PhD project was designed the distribution of viral reservoirs over body sites and its poor reflection by measures in peripheral blood was already acknowledged, but not yet extensively explored. Thus as a first objective of the project we intended to study viral reservoirs in peripheral tissues of humanized mice. By the time the mouse model was established in our laboratory, CD32 had been postulated as a marker of the replication competent reservoirs, based on data from peripheral

blood lymphocytes (11). As no information on its role in lymphoid organs was available at that time we included this question as a central aspect into the study.

Many studies have been characterizing T cell responses as potential correlates of protection, with often inconclusive or contradictory results. From the literature of natural controllers, a consistent finding is that their CD8+ T cells have a superior viral suppressive effector function freshly *ex vivo.* The precise phenotype of the virus suppressive cells and their "memory" function (i.e. their capacity to expand and functionally mature upon appropriate antigenic stimulation), however, have not been studied in depth. Thus, the second main element of this PhD work was to identify detailed phenotypes of CD8 + T cells associated with increased viral suppression *in vitro*. To do so we established two cohort studies collecting samples from different groups of natural controllers and treated progressors. These data might serve as correlates for future clinical vaccination strategies.

RESERVOIR DYNAMICS AND BIOMARKERS IN VIRALLY SUPPRESSED HUMANIZED MICE

Since 2015, HIV-1 treatment guidelines stress the importance of starting ART as early as possible to reduce AIDS related and non-AIDS related events (12) as well as new infections regarding treatment as prevention. Moreover early therapy initiation decreases the total HIV-1 reservoir significantly in size (13). However, many aspects of treatment and reservoir dynamics remain to be understood especially in peripheral tissues. In this respect, animal models provide selective advantages over clinical samples. They allow access to all body tissues but also precise control over the timing and duration of treatment. Accordingly, the project aimed at a thorough investigation of HIV-1 infected and ART treated mice with three aspects in particular:

- Does early treatment result in a delay of viral rebound after treatment interruption as compared to late treatment initiation?
- Does timing of treatment initiation impact reservoir size and immune activation in peripheral tissues?
- Does CD32 represent a biomarker for the HIV-1 reservoir in tissues of virally suppressed mice?

Concerning the first aspect, our data shows no difference in time to viral rebound after treatment interruption comparing early versus late treated mice. The VISCONTI trial has reported on 14 post treatment controllers or PTC in which treatment initiation during primary infection was identified as common factor (14). Some studies have reported similar data (15,16), whereas other analytical

treatment interruption (ATI) trials observed rapid viral rebound despite treatment initiation during acute infection (17,18).

Intriguingly, we measured a significant reduction in total HIV-1 DNA of early treated mice, while still virally suppressed, in most body tissues, comparable in magnitude to what is commonly reported for post-treatment controllers (PTC) from peripheral blood (19). Having said that, it's even more astonishing that despite this clear reduction in size over most body reservoirs no difference in the median speed of viral rebound was observed. Importantly these mice have a reduced anti-viral CD8 + T cell efficacy due to defective T cell education in the context of murine MHC-I only (20). Hence, less selection pressure during the establishment of reservoirs but at the same time lower viral containment while reactivation could hypothetically explain effects in both ways. Importantly though on the individual level we observed several mice of the early treated group, trending back to un-detectability whereas none of the late treated animals did. Overall, our data suggests that total HIV-1 DNA is not an adequate marker of viral rebound even when assessed in tissues and under conditions of fully controlled treatment. In perspective, a readout or marker for post treatment control or PTC needs to be defined. Exploring in the future to what extent the integrity of proviral DNA, the efficiency of viral transcription and replication as well as the effective anti-HIV-1 immune responses plays into PTC is highly important for the field.

Next, we observed normalization of CD4 + T cell counts and a significant reduction of immune activation after ART initiation, as was expected. This effect was more pronounced by early ART and emphasizes the importance of early viral suppression. During the recent years, studies have underlined the effect of defective provirus on persisting immune activation in humans under therapy. Although those sequences cannot deliver functional viral progeny, they can be transcribed into (defective) RNA and the expression of these viral RNA species triggers host-defense pathways fueling chronic immune activation (21–23). We did not perform proviral sequencing but the enormous reduction of immune activation during viral suppression by early ART allows to hypothesize that the total pool of functional and defective provirus is reduced. Taken together we can add clear arguments for the benefits of early treatment initiation concerning immune activation. It would be interesting to assess in this model to what extent defective proviral sequences under suppressive ART drive persisting immune activation.

Our understanding of the enormous heterogeneity of latently infected cells keeps evolving. Concisely, latency is no longer regarded as a complete lack of viral transcription, but rather as a continuous spectrum of blocks at different stages of viral transcription installed in various cell population (24)

(chapter 2.9). Hence, it is quite possible that abortive viral RNA transcription induces host proteins, some of which might be expressed at the cell membrane. Therefore, a signature surface marker to identify latently infected cells is a long-standing objective in the field. A controversial debate has started since CD32 was proposed as a such a biomarker for the HIV-1 reservoir (25). Shortly thereafter, we conceived our project and decided to investigate the potential of CD32 to identify tissue reservoirs of virally suppressed mice. From our view, this should add valuable information into the debate because most studies are limited to the peripheral blood. While our project was running, several studies reported divergent findings which are likely driven by the technical difficulties to obtain pure CD4 + CD32 + T cells (extensively reviewed in (26)). We had strict procedures in place during flow sorting and flow phenotyping to preclude any bias (see the material and methods of chapter 4 for technical details).

We find indeed that CD32 expressing CD4+ T subsets were enriched for HIV-1 DNA in all tissues. On the other hand though they are neither more productive during therapy nor the major contributor to viral replication after latency reversal. Interestingly recent studies on human lymph nodes localize CD32 + subsets mainly in the B cell follicles with transcriptionally active HIV-1 (27,28). It has to be emphasized that the angle from where you look at it can changes it all. This is intriguingly clear from our data on P24 expression. While P24 producing cells are increased within the pool of CD32 expressing memory T cells they represent only a minor fraction of total P24 producing reservoirs. In the future it might help to better understand CD32's functional implication especially in the light of labelling a highly activated and hence rather short-lived reservoir (29). Consequently its persistence depends most likely on continuous proliferation which in turn could allow an interesting angle to better understand reservoir proliferation as a crucial aspect in HIV-1 long term maintenance (30).

In conclusion, there is still a lot to understand about which markers label the major viral reservoirs. In a nutshell what we could call a complexity escalation loop, the more variables we start to understand the more are to be considered. This is well reflected by the rapidly growing literature of CD32 in HIV reservoirs, which has been initially described only about two years ago. Maybe a semantic bias begins by using the term reservoir in singular. The existing literature and our own data explicitly prove: there is a heterogeneous continuum with many faces (i.e. different levels proviral sequence integrity, different basal transcriptional activity) and hence no such single entity as the reservoir (that's why I keep pushing myself to use the plural of reservoirs while writing). From this follows that there is very probably also no single marker for all of them. More probably a combination of several molecules will allow to clearly allocate HIV-1 reservoirs in CD4 + T cells.

In analogy to what was once called coding versus junk DNA in the human genome, the clinical relevant reservoir is most often defined as those intact proviral sequences able to produce fully functional viral progeny. While this holds true in the perspective of viral rebound, more and more literature revisits the importance of defective viral sequences especially on persisting immune activation (22). Ironically, these are likely the most clinically relevant reservoirs for HIV-1 pathogenesis in the era of ART based healthcare, because most well-treated patients today rather suffer from non-AIDS morbidities caused by chronically heightened immune activation (31).

VIRAL SUPPRESSION BY CD8 + T CELLS AND IMMUNE CORRELATES IN TREATED PROGRESSORS

Anti-viral CD8 + T cell immunity is thought to be a main factor in long-term natural control over HIV-1 (32). These observations inspired clinical trials in the search for a prophylactic vaccination such as the STEP study (33). Clearly, in the field of prophylactic vaccination, HIV-specific T cell responses are insufficient and antibody-mediated mechanisms of protection are needed. Conversely, there is consensus today that cell-mediated immune response is important for a therapeutic vaccination/intervention in view of "functional cure" (34). Consequently boosting of antiviral CD8 + T cell immunity is a valuable element to increase host mediated clearance in approaches such as "shock and kill". In practice though there is still to date no ideal assay to assess the efficacy of such intervention *in vitro.* Assays such as IFN-γ ELISPOT and intracellular cytokine staining have proven limited use as predictors of *in vivo* control (35,36). In contrast viral inhibition assays or VIA measure direct CD8 + T cell mediated inhibition of viral outgrowth hence might deliver stronger correlates of clinical relevance (37,38). To this end, the project aimed at answering three main questions:

- Is viral suppressive capacity (VSC) present in well-treated progressors?
- Can HIV peptide stimulation boost VSC?
- Are there clinical or immune correlates with increased VSC?

First a reproducible VIA was setup. We considered a low background as an important asset to the identification of clinically relevant correlates. This was achieved by omitting IL2 during pre-culture of CD8 + T cells resulting in a low background of HIV-1 (-) healthy controls. In the ART treated patients VSC was expectedly limited during unstimulated condition. Interestingly it increased significantly upon consensus Gag peptide stimulation. Likely, the HIV-1 peptides are presented by antigen presenting cells or APC to the T cells leading to their expansion and activation. This proved that the assay is sufficiently sensitive to discriminate reinvigorated T cell responses while showing low background at the same time.

Intrigued by the clear increase in VSC after peptide stimulation, which can be considered as an *in vitro* analogue of *in vivo* (therapeutic) vaccination, we wondered what discriminates these suppressors from non-suppressors. When compared to non-supressors we found that the co-expression of PD1 and CD160 was significantly increased on terminal differentiated memory CD8 + T cells (so called TEMRA) of suppressor patients. In addition, was CD57, a protein linked to terminal differentiation and replicative senescence, higher expressed on memory CD8 + T cells. Under the hypothesis that these cells are the pivotal players in HIV-1 containment, two main insights can be gained from this data.

The first concept concerns immune exhaustion. PD-1 is maybe the most studied and prominent representative of molecules from the family of immune checkpoints. First identified in chronic viral infections and found to be highly expressed and causative for an impaired T cell response, termed immune exhaustion (39). The enormous potential abolishing the signaling of these negative T cell regulators is illustrated by the therapeutic success of “checkpoint blocking” antibodies in cancer immunotherapy (40). Consequently, the identification of subsets with co-expression of several immune checkpoint molecules on CD8 + T cells of HIV suppressors is very surprising at first. However during ART a general decrease of PD-1 expression is observed and low viremia of our patients makes chronic exhaustion unlikely (41). Also could those receptors testify activated subsets fighting virus during treatment (42). In any case, it is worthwhile to stick to the term of immune checkpoints when referring to those molecules. This because their expression can be viewed as a physiological feedback precluding potential harm by never ceasing cytotoxic T cells during constant presence of the antigen (43). Hence, it makes sense to refer to immune exhaustion only in the context of chronic antigenaemia such as during persistent high pVL in untreated HIV-1 or in the tumor micro environment (44).

The second concept addresses immune senescence. Aging leads to profound physiological changes among the most striking observed in the cellular immune system due to lifelong antigenic stimulation (chapter 2.12.5). Concomitant goes a low-grade systemic inflammation (i.e. elevated circulating cytokines) considered the leading cause of age related morbidities including cardiovascular, neurodegenerative and metabolic disease (45). Fact is that virally suppressed HIV-1 infected patients are still at higher risk for age associated disease than healthy controls (46,47). However, it is not clear to what extent this links with cellular senescence on HIV-1 specific CD8 + T cells. Recurrent latent infection such as CMV can drive proliferative senescence in T cells which associates with CD57 expression (48). Although those subsets are arrested in proliferation, CD57 + CD8 + T cells show strong cytotoxic functioning (49). It appears thus from our observation and others that CD57 expressing T cells harbor strong antiviral capacities (50–52). To what extent these terminal differentiated T cell

subsets are able to maintain *in vivo* control remains to be defined. Interestingly a recent report links cytolytic CD4+ T cells expression of CD57 in elite controllers or EC (53). Future interventions to address shortened telomeres in these cell types could overcome proliferative arrest and increase their effectiveness further (54).

In summary, our study adds important findings into the picture of viral inhibition during antiviral therapy and the potential to reactivate particular CD8+ T cell subsets for more efficient HIV-suppression by antigenic stimulation. The editorial comment inspired by our paper nicely elaborates on potential non-cytolytic factors involved (55).

CD8 + T CELL FUNCTIONING DURING NATURAL CONTROL – A MATTER OF MULTIFUNCTIONALITY

The best model for CD8 + T cell mediated viral control remains the clinical phenotype of long-term non-progression (chapter 2.12). After we had observed increase of viral suppression by peptide stimulation in treated progressors a thorough comparative analysis with natural controllers was needed in order to define correlates with *in vivo* control. We sought to answer:

- Does VSC differ between controllers and (treated) progressors?
- Can Gag stimulation level out VSC between controllers and progressors?
- Are there distinct traits and subsets of antiviral CD8 + T cells which relate to VSC during natural HIV control?

Clearly, controllers showed stronger VSC, at baseline and after pre-stimulation. Again though, as observed in our previous study, Gag peptide stimulation did boost VSC significantly in progressors as well. The most striking distinctive feature of natural controllers was the existence of multiple polyfunctional CD8 + T cell subsets, which correlated convincingly with VSC. The ultimate proof for their capacity would be gained by sorting and subsequent viral inhibition assay or VIA. Furthermore, single cell transcriptomics could allow to identify the active gene program of such signature subsets. An important step to understand how to induce them.

Overall we know since long time that there is increased quality of antiviral CD8 + T cell responses during elite control of HIV-1 (chapter 2.12) hence it was not the central point to re-emphasize this. However, in a setting of therapeutic vaccination clear correlates of the cellular phenotype with viral suppression are missing. This in order to evaluate the potential of tested regimens *in vitro* and to distinguish the spectrum of VSC observed among patients from potential *in vivo* control. In addition, could this

knowledge aid to monitor CD8 + T cell activity after therapeutic vaccination as a signature of a potent T cell response.

It is worthwhile to underline the observed multiplicity of cytotoxic CD8 + T cell subsets in terms of tissue homing. It follows that homing of highly cytotoxic subsets into central lymphoid organs, to the periphery but also potentially into the B cell zone of lymph follicles is a distinctive capacity of CD8 + T cells during elite control or EC. Interestingly it has been repeatedly shown that the lymphoid follicles represent important sites of reservoir persistence (56). Therefore a better understanding of the lymph node microenvironment (LNME) and its impact on the functionality of CD8 + T cells will offer many prospect angles for intervention (similar as happened for the tumor microenvironment over the last decade). Mechanistically a recent study has underlined that elite controllers succeed to establish an immunological synapse even with resting CD4 + T cells (57). Clearly, this means that CD8 + T cells of controllers likely nip things in the bud – kill latently infected cells even before they are strongly reactivated and hence productive.

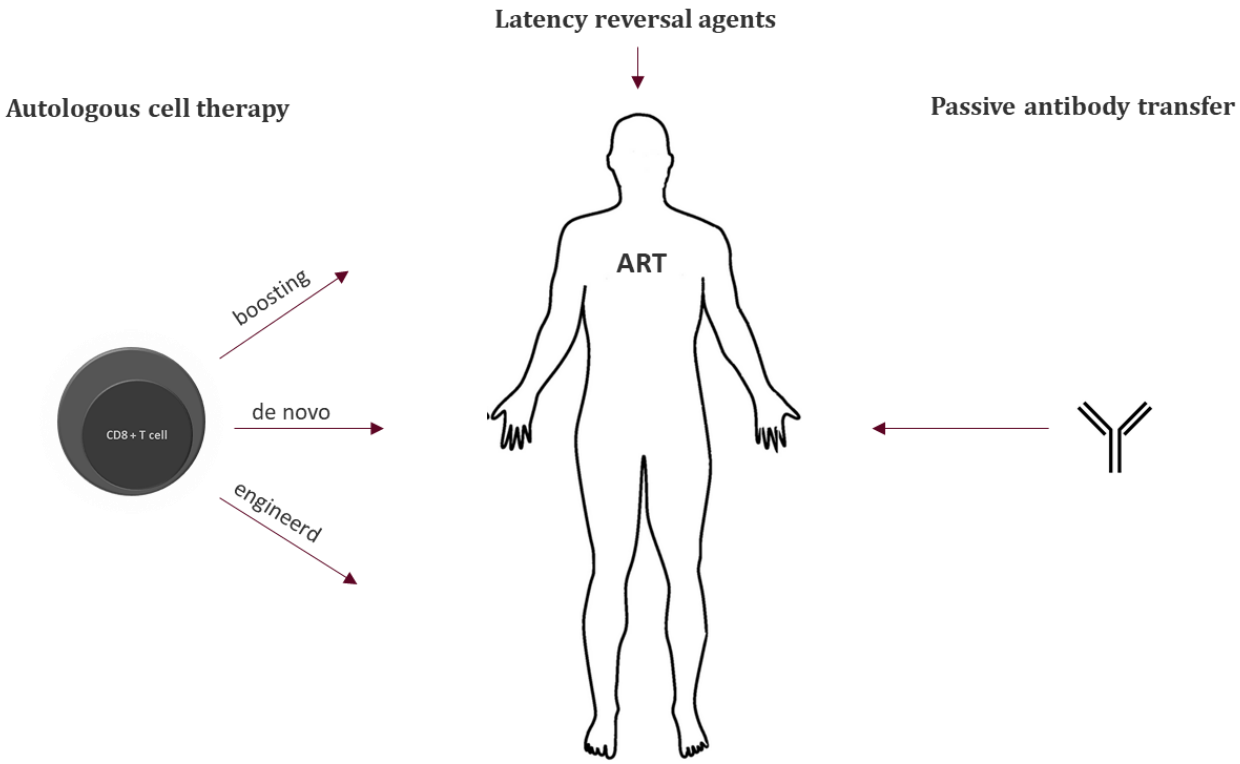

Figure 17. A care model for a functional cure of HIV-1
Currently four elements appear central to achieve a HIV-1 functional cure. ART suppressed viremia forms the backbone of clinical care. Under stable therapy, the use of latency reversal agents to purge viral reservoirs can be combined with passive antibody transfer (especially broadly neutralizing entities) and autologous cell therapy to maximize the immune mediated clearance of HIV-1 reactivated cells. Our work specifically emphasized the expansion of distinct polyfunctional anti-viral CD8 + T cells subsets be it by boosting of existing memory responses, induction of the novo responses or engineerd strategies such as CAR T cell, for viral control.

A deeper understanding of these mechanics is mandatory as instant vigorous cytotoxicity, precluding viral replication is likely a crucial element of how to control. In this context it remains to be underscored that the rapid presentation of epitopes in the context of HLA-I is likely an intrinsic advantage for immune recognition at early stages of viral reactivation. In summary the presented data makes us to be strongly convinced that the key to ART free remission lies in the access of potent CD8 + T cells to immune sanctuary sites of reservoir persistence.

Current ART medication is highly effective. At the same time, the ART based healthcare has many gaps to close, as will be discussed in the last paragraph. Why then should a cure be of priority? Briefly, the success of ART leads to globally growing number of patients in need for life-long drug intake with possible side effects and persisting heightened inflammation. Consequently, cure research is essential to find an answer to the growing needs of HIV-1 pathogenesis under ART. A big research community over various areas has been dedicated to HIV-1 cure in the recent years. This alone keeps the hopes high that a cure is in reach. Main efforts around the characterization and quantification of reservoirs will lead to a clear understanding how latency is established, maintained and potentially reverted (58).

In the context of “shock and kill” the improvement of latency reversing agents or LRA is a top priority and will enable new analytical treatment interruption trials (59). Also will the use of antibodies against immune checkpoints be assessed to increase T cell mediated clearance of reactivated cells (60). In the same line value comes likely also from advances of engineered (CAR T cells) T cells recognizing HIV peptides(61). An interesting approach is pursued by the use of broadly neutralizing antibodies to induce CD8 + T cell immunity (7). Apart from that the “block and lock” strategy experiments with induction of “deep latency” by tat inhibitors (62). Here again gene therapy is being explored to excise integrated proviral sequences with the possibility to clear latency from the host without the need for reactivation (63).

In perspective, given the complex picture of viral latency, likely a combination of several strategies will lead to success. This implies at the same time that choices from a spectrum of interventions become available allowing tailored cascades of a cure one day. Especially against the background of a global enormously heterogeneous group of patients, i.e. age, ART regimens or comorbidities, this variety of promising research lines keeps the hopes high.

PRIORITIES AND OPPORTUNITIES TODAY IN GLOBAL HIV-1 CARE

A frequent reaction when questioned from outsiders about this field of research is something like, “That’s interesting but is there not already an effective treatment available for HIV?” or scientists from other domains might say, “ART does the job, so why actually looking for more, what’s the added value?”. Profound changes in the medical care of HIV-1 might have been accompanied with a shift in the public perception. The haunting fear of a killing virus was taken from us – did it really?

Worldwide more than 15 million people live with HIV-1 having no access to ART (UNAIDS). This means high viral loads, high risks of transmission and high chances of AIDS related death. Globally 2 million get newly infected and 1 million die – every year (UNAIDS). Whereas a cure is a call for the future HIV-1 is still a global health emergency. As outlined in the introductory chapter the current UNAIDS objectives stress a need for improvement on all three levels – diagnosis, treatment access, and viral suppression to reach up to the 90-90-90 goals, which will not be reached by 2030.

Much progress in HIV-1 surveillance has been made. Scale up of testing has achieved that 79% globally are aware of their status in 2018 (UNAIDS) (64). Much of its progress is attributed to provider-initiated testing and counseling as well as the introduction of more community based testing services (65). Especially in Sub-Saharan Africa a major challenge remains the continuous search for the millions unaware of their status. Due to funding shortage and focus on the yield, referring to the proportion of tests being positive, the 2020 goals are not achieved yet (66).

In 2018, 62% of the globally nearly 37 million people living with HIV had access to treatment. The most important barriers for a further increase are linked to build up of human resource capacities and education as reflected by fear about HIV stigmatization, HIV-1 denial and ART side effects (67,68).

Viral load monitoring is a crucial aspect to ART based healthcare as low treatment adherence or viral escape mutations can only be detected by regular surveillance. Studies report that viral failure (defined as pVL > 1000 copies/ml) is frequently not followed up by adherence counseling or appropriate follow up tests (69,70). Further alarming evidence states that the time to treatment switch after confirmed viral failure is 68 weeks (71). Obviously, under these circumstances of prolonged “suboptimal” regimen, HIV strains with multiple drug resistance mutations will emerge and spread in the local communities and eventually worldwide, thus threatening the efficacy of present drug regimens on the longer run.

Undoubtedly, parts of the mentioned programmatic failures are symptomatic of healthcare systems in low and middle income countries. This makes HIV-1 a mirror of socio-economical circumstance and emphasizes need for further developmental aid. New technologies as eHealth offer opportunities to strengthen global surveillance and reach out to patients. Further great opportunities in that matter are long acting and extended release antiretrovirals (72–74). These medications will help to improve adherence and protection by omitting the need of daily drug intake and decrease finally stigma. Taken together HIV-1 clearly remains a highly complex global healthcare priority with many gaps to close. These gaps will imply to develop novel multidisciplinary approaches to reform the testing strategy (in order to reach the key individuals for HIV transmission), to restructure the health care system as well as to reduce stigma.

Is in the public perception the fact of incurability of HIV-1 a last strong anchor point to raise attention for the unmet needs outside the western world? Would Tom Hanks walk the streets of Philadelphia on ART?

These questions intend point to the power and importance of awareness. Awareness of the unmet global needs to acquire more funding. Awareness to spread the word of the effects and limitations of ART. Awareness for prevention. Awareness for the ongoing social and psychological impact to fight stigmatization. In that respect recent campaigns as the U=U (undetectable equals untransmittable) are essential to transport a key message: therapeutic adherence allows safety and relieves stigma (75).

Overall, a complex field as HIV/AIDS pandemic demands several priorities. Whereas a cure is of relevance for the future of HIV-1 care today of utmost importance stands the stable global implementation of the ART based treatment cascade.

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SUMMARY

Antiretroviral therapy (ART) halts progression to AIDS but is not curative due to persisting viral reservoirs in CD4 + T cells. Therefore patients depend on lifelong drug intake accompanied with potential side effects, drug resistance and heightened chronic inflammation. Besides the high economic costs, reach out to patients is especially challenging in low and middle income countries, which are most, affected from HIV-1. In addition to the need for increase of ART coverage in parts of the world, we face a growing number of a chronically infected and aging patient population worldwide. Consequently, new curative approaches to end HIV-1 infection are needed.

The total removal of HIV-1 reservoirs from the body ("sterile cure") has proven impractical to date due do its high diversity and early seedings in tissues. However, ART free remission while HIV-1 remains detectable, termed a functional cure, might be in closer reach. In any of both cases, the search for exclusive markers of the replication competent viral reservoirs remains valuable with the ultimate objective of targeted depletion.

Most studies in humans are limited to the reservoir in the peripheral blood. HIV-1 infected humanized mice can help to gain insight into the tissue reservoirs. In this context, elite controllers, characterized by a very low viral reservoir, represent a rare and unique profile of natural control with opportunities to study immune-mediated containment of HIV-1 replication. Under the current consensus that a combination of interventions depleting the reservoir and improving host mediated clearance would most likely lead to viral control, the objectives of this PhD project were divided in two interrelated thematics: The characterization of tissue HIV-1 reservoirs in humanized mice and the identification of $CD8^+$ T cell subsets associated with improved *in vivo* control of HIV-1.

Viral reservoirs are diverse entities with regard to which tissues and CD4 + T cell compartment they reside in. Therefore we sought to investigate reservoir dynamics in a well-controlled setting of early and late ART initiation in HIV-1 infected humanized mice. A particular focus point was the role of CD32, a recently proposed marker for the replication-competent reservoir in human blood CD4 + T cells, which we now wanted to investigate in peripheral tissues of virally suppressed animals. Clearly, early treatment significantly reduced total HIV-1 DNA whereas we observed no initial changes in viral rebound between early and late treated animals. However, over 25 % of early treated (and none of the late treated) animals went back to undetectable viral load after a short period of viral rebound. Interestingly $CD32^+CD4^+$ memory T cells were enriched for total HIV-1 DNA but not for cell-associated

early RNA transcript levels. Importantly $CD32^+CD4^+$ memory T cells showed also a relative enrichment in productive infection upon latency reversal but constituted a negligible compartment for the total replication competent reservoir.

The viral suppressive capacity (VSC) of $CD8^+$ T cells from patients under ART is generally found to be low. In order to test approaches of therapeutic vaccination we investigated HIV-1 consensus Gag peptide stimulation to increase viral suppression *in vitro*. Interestingly about 60% of ART patients indeed showed a considerably higher peptide induced VSC, which was associated with an expanded CD8 + memory T cell subsets co-expressing the immune checkpoint molecules, PD1 and CD160. Furthermore, upregulation of CD57 and HLA-DR expression were associated with increased *in vitro* suppression. These findings indicate a potential role of terminally differentiated $CD8^+$ memory T cells with regard to efficient HIV-1 suppression in ART patients.

Spontaneous controllers of HIV-1 infection are recognized for their highly functional $CD8^+$ T cell responses. However the exact subsets most effective in viral suppression have not been defined yet. To this end, the last part of this project intended to define immune correlates of natural control mediated by HIV-1 specific $CD8^+$ T cells. Upon stimulation with potential T cell epitope (PTE) Gag peptides, viral suppressive capacity was strongly upregulated in controllers and this correlated with increased CXC5 expression, pointing to the capacity of these cells to home in germinal centers of the lymphoid organs, which are known as “sanctuar” HIV reservoirs. Furthermore, several distinct multifunctional cytotoxic subsets which correlated well with viral suppression were identified. These findings underline the probable role of distinct multifunctional $CD8^+$ T cell subset with tissue homing abilities during natural control of HIV-1.

In conclusion, our studies contribute to mapping the HIV reservoir distribution under antiviral therapy. Moreover, we also show that antigenic stimulation with Gag induces multifunctional CD8 T cell responses with the potential ability to suppress HIV replication in the lymphoid tissues. These new insights will need to be translated into therapeutic strategies for functional cure of HIV-1.

SAMENVATTING

Antiretrovirale therapie (ART) stopt de progressie naar AIDS maar is niet curatief vanwege persisterende virale reservoirs in CD4 + T-cellen. Daarom zijn patiënten afhankelijk van levenslange inname van geneesmiddelen, hetgeen gepaard gaat met mogelijke bijwerkingen, resistentie tegen geneesmiddelen en verhoogde chronische ontsteking. Naast de hoge economische kosten, is het vooral een uitdaging om patiënten te bereiken in landen met een laag en gemiddeld inkomen die het meest getroffen zijn door HIV-1. Enerzijds is er nood aan verder uitrollen van antivirale middelen in grote delen van de wereld. Anderzijds worden we geconfronteerd met een wereldwijd groeiend aantal chronisch geïnfecteerde en verouderende patiënten. Bijgevolg zijn nieuwe curatieve benaderingen om HIV-1-infectie te beëindigen echt wel nodig.

De totale verwijdering van HIV-1-reservoirs uit het lichaam ("steriele genezing") is tot op heden onmogelijk gebleken vanwege de hoge diversiteit en vroege uitzaaiing van het reservoir in allerlei weefsels. Een remissie zonder therapie terwijl HIV-1 detecteerbaar blijft als provirus, ook wel een "functionele genezing" genoemd, lijkt een meer haalbare mogelijkheid. Een voorwaarde om "genezing" te monitoren is het identificeren van exclusieve "biomerkers" van de replicatie-competente virale reservoirs. De meeste studies in mensen blijven beperkt to het reservoir inhet perifere bloed. Gehumaniseerde muizen kunnen een hulp zijn om ook zicht te krijgen op de weefselreservoirs.

In deze context vertegenwoordigen ook elite-controllers, gekenmerkt door een zeer laag viraal reservoir en een krachtige virus-suppressieve immune respons, een interessant model om "functionele genezing" te begrijpen. De huidige consensus is immers dat een combinatie van interventies gericht op uitputting van het reservoir enerzijds en de verbetering van door de gastheer gemedieerde klaring anderzijds, nodig zal zijn voor "functionele genezing". Daarom liggen twee gerelateerde onderzoeksthema's voor de hand: de betere karakterisering van HIV-1-reservoirs in de weefsels van gehumaniseerde muizen en de identificatie van CD8 + T-cel subsets geassocieerd met verbeterde in vivo controle van HIV-1.

De eigenschappen van virale reservoirs kunnen verschillen naargelang de weefsels en het CD4 + T-celcompartiment waarin ze zich bevinden. Daarom hebben we geprobeerd om de reservoirs te onderzoeken in een goed gecontroleerde setting met vroege versus late start van antivirale behandeling bij met HIV-1 geïnfecteerde gehumaniseerde muizen. Een specifiek aandachtspunt was de rol van CD32, een recent voorgestelde marker voor het replicatie-competente reservoir in CD4 + T-

cellen van menselijk bloed, dat we nu wilden onderzoeken in perifere weefsels van deze dieren met een behandelde HIV infectie. Het werd duidelijk dat vroege behandeling het totale HIV-1-DNA aanzienlijk verlaagde in allerlei weefsels, terwijl desondanks het onderbreken van zowel vroege als late behandeling aanleiding gaf tot een snelle virale rebound zagen. Echter, meer dan 25% van de vroeg behandelde (en geen van de laat behandelde) dieren gingen na een korte periode van virale rebound terug naar niet-detecteerbare virale lading. Interessant genoeg bleken CD32 + CD4 + geheugen-T-cellen aangerijkt voor totaal HIV-1-DNA maar niet voor cel-geassocieerde vroege RNA-transcripten. Belangrijk is dat CD32 + CD4 + geheugen T-cellen ook een relatieve verrijking vertoonden in productieve infectie na in vitro stimulatie, maar desondanks vormen ze maar een verwaarloosbaar klein deel van het totale replicatie-competente reservoir.

De “virale suppressieve capaciteit” (VSC) van CD8 + T-cellen van patiënten onder ART blijkt over het algemeen laag te zijn. Om een *in vitro* model van therapeutische vaccinatie te testen, hebben we lymfocieten met HIV-1 consensus Gag peptiden gestimuleerd en het effect op de VSC van CD8+ T cellen onderzocht. Interessant genoeg vertoonde ongeveer 60% van de ART patiënten inderdaad een aanzienlijk hogere door peptide geïnduceerde VSC en deze functionele verandering was geassocieerd aan verschillende CD8 + geheugen T-cel subsets die de immuun checkpoint-moleculen, PD1 en CD160, tot expressie brengen. Verder werden CD57- en HLA-DR-expressie ook selectief opgereguleerd tijdens verhoogde *in vitro* VSC. Deze bevindingen wijzen op een mogelijke rol van terminaal gedifferentieerde CD8 + -geheugen T-cellen in efficiënte hun HIV-onderdrukkende functie.

Patiënten die spontaan (zonder behandeling) HIV *in vivo* controleren, worden gekenmerkt door hun zeer functioneel active CD8 + T-cellen met hoge virale suppressieve capaciteit. De exacte subsets die het meest effectief zijn bij virale onderdrukking zijn echter nog niet gedefinieerd. Daarom probeerden we in het laatste deel van dit project de immuun correlaties te definiëren van deze spontane HIV-1 specifieke CD8 + T-cel activiteit. Na stimulering met “potential T epitoop” (PTE) Gag peptiden stelden we een sterke verhoging van het virale suppressieve vermogen vast bij “controller” patiënten, gecorreleerd met de expressie van de CXCR5, die typisch is voor CD8+ T cellen, die naar de germinale centra van de lymfoïde organen migreren, waar zich een belangrijk deel van het HIV reservoir bevindt. Ook verschillende andere multifunctionele cytotoxische subsets correleerden met virale suppressie. Deze bevindingen onderstrepen de rol van verschillende multifunctionele CD8 + T-cel subset met affiniteit voor de lymfoïde weefsels tijdens natuurlijke controle van HIV-1.

In conclusie: onze studies leveren een bijdrage aan het in kaart brengen van de HIV reservoir distributie onder antivirale therapie. Anderzijds tonen we ook aan dat antigenische stimulatie met Gag multifunctionele CD8 T cel responses induceert met het vermogen om HIV replicatie ook in de lymfoïde weefsels te onderdrukken. Deze nieuwe inzichten zullen hopelijk vertaald worden in therapeutische strategieën voor functionele genezing van HIV-1.

ANNEX

Additional published output during the doctoral thesis:

"T Cell Immunosenescence after Early Life Adversity: Association with Cytomegalovirus Infection" (Frontiers of Immunology, 2017) Elwenspoek MMC[1,2], Sias K[1], Hengesch X[3], Schaan VK[4], Leenen FAD[1,2], **Adams P[1]**, Mériaux SB[1], Schmitz S[1], Bonnemberger F[1], Ewen A[1], Schächinger H[3], Vögele C[4], Muller CP[1,2], Turner JD[1].

Multicenter evaluation of the cobas® HIV-1 quantitative nucleic acid test for use on the cobas® 4800 system for the quantification of HIV-1 plasma viral load. (Journal of clinical virology, 2019) **Adams P[1]**, Vancutsem E[2], Nicolaizeau C[1], Servais JY[1], Piérard D[2], François JH[3], Schneider T[4], Paxinos EE[5], Marins EG[5], Canchola JA[5], Seguin-Devaux C[6].