Cytolytic T cells in chronic HIV-1 infection

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Well, if you want to sing out, sing out

And if you want to be free, be free

'Cause there's a million things to be

Cat Stevens

All the experiments for this study have been carried out at the Institute for HIV Research, University Hospital Essen, at the University of Duisburg-Essen or at another equivalent facility.

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Die Experimente der vorliegenden Arbeit wurden am Institut für HIV Forschung, der Universität Duisburg-Essen oder an einer anderen gleichwertigen Einrichtung durchgeführt.

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Inhaltsverzeichnis

1 Introduction

1.1 HIV

The first documented cases of HIV were reported in 1981. The Centers for Disease Control and Prevention (CDC) described five unusual cases of a rare lung infection with *Pneumocystis carinii pneumonia* and at the same time, a group of men was diseased with the very aggressive cancer Kaposi´s Sarcoma (1, 2). By the end of the year, there were 270 reported cases of severe immune deficiency among gay men in the USA and in 1982, the symptom complex was named AIDS (acquired immune deficiency syndrome) (3). The first virus isolation was performed in 1983 at the Pasteur Institute in France and one year later at the National Cancer Institute in the USA (4). Both announced the cause of AIDS was the retrovirus human T-lymphotropic virus, type 3 / lymphadenopathy-associated virus (HTLV-III/LAV), which was changed to human immunodeficiency virus (HIV) in 1986 (5).

Since then, HIV has caused the death of more than 35 million people and currently 37 million people are living with HIV, of whom 51 % are women with the highest burden in Eastern and South Africa (**Figure 1**) (6, 7). Yearly there are 1.7 million new infections and nearly 690.000 people are still dying every year on AIDS-related causes (6). So far, there is no vaccine available but in the early 1990s, the U.S. Food and Drug Administration (FDA) approved the first antiretroviral drug: Zidovudine (AZT). The introduction and refinement of antiretroviral therapy (ART) changed the face of HIV and AIDS from a usually deadly infection to a manageable chronic disease (8). The life expectancy increased quickly from 6-10 years to a normal life span (9).

In 2015, the United Nations Program on HIV/AIDS (UNAIDS) presented the 90-90-90 strategy to end the AIDS epidemic and decrease the number of new infections by the year 2020. UNAIDS's 90-90-90 goals set as targets that by 2020, 90% of all people living with HIV will know their HIV status, 90% of all people diagnosed with HIV will receive suitable ART, and 90% of all people on ART treatment will have complete viral suppression (10). Regardless of all efforts, in 2018 of all people living with HIV, 79% knew their status, 62% were accessing treatment and only 53% were virally suppressed (10). The reason for that is mostly limit access to treatment in developing countries.

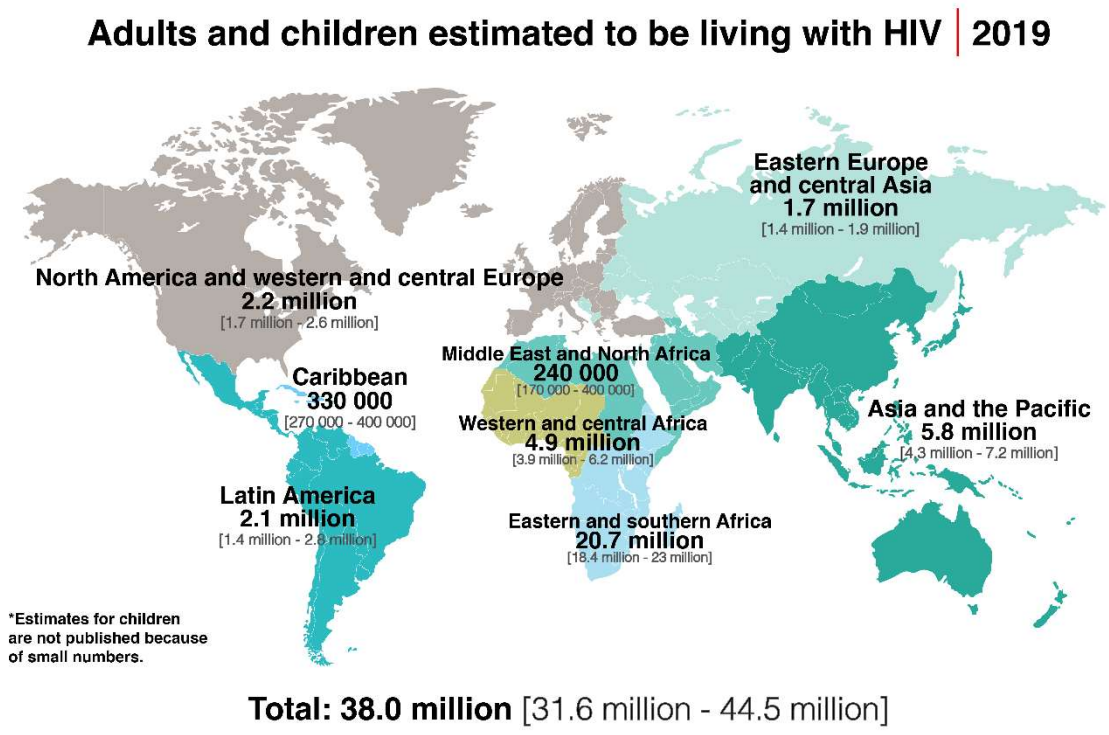

Figure 1: People estimated to be living with HIV in 2019 (figure adapted from: (11)).

1.1.1 Structure of HIV-1

HIV is grouped to the genus *Lentivirus* within the order *Ortevirales* and the family of *Retroviridae*, subfamily *Orthoretrovirinae (12)*. Further HIV can be divided into two major types, HIV-1 and HIV-2. Additionally, HIV-1 can be branched out into different groups (M, N, O, P), although group M is the most common type and therefore subdivided into subtypes (A to L). Each subtype is dominantly represented in different parts of the world; subtype B is the most common form in Europe (12, 13). The structure of the virus shows a lipid membrane from the host-cell origin, which surrounds HIV-1. Located on the surface of the lipid membrane are two noncovalently associated glycoproteins gp120 and gp41 (**Figure 2**). Both are generated by proteolytic cleavage of the precursor polypeptide gp160. The gp120 subunit is anchored to the membrane by the trimers of the transmembrane protein gp41. The inner part of the viral membrane is covered with the HIV-1 matrix, which is composed of an association of the viral matrix protein p17. Both, viral membrane and matrix, surrounds the capsid, which is usually an asymmetric cone formed by capsid proteins p24 and encapsulates two copies of the viral genomic RNA. It also contains three essential viral enzymes encoded by the virus: protease, reverse transcriptase, and integrase (14-17).

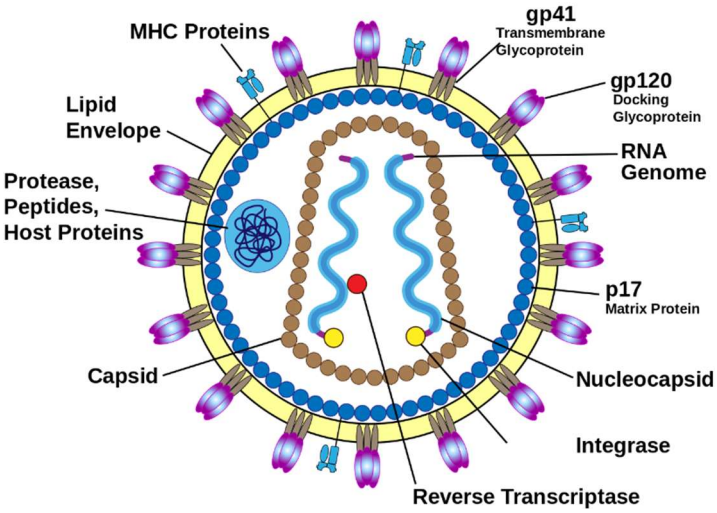

Figure 2: Schematic structure of HIV-1 (figure from: (18)).

1.1.2 Replication of HIV-1

HIV-1 is able to infect first dendritic cells or macrophages as well as monocytes after transmitting via sexual contact. However, the main target of HIV-1 are CD4 T cells, especially the subset of T follicular helper cells (12). In order to infect these target cells, HIV-1 binds with the gp120 to the CD4 protein on the surface of the cell (**Figure 3**). This binding causes a conformational change in the envelope complex, which promotes the interaction of gp120 with specific co-receptors (CCR5 or CXCR4) on the surface of the target cell. The interaction of gp120 with the CD4 protein, together with CCR5 or CXCR4 is required for the fusion of cellular and viral membranes (19-21). After the fusion HIV RNA, reverse transcriptase, integrase and other viral proteins enter the host cell. The reverse transcriptase converts viral RNA into proviral double-stranded DNA that interacts with viral and cellular proteins to form the pre-integration complex (PIC). Therefore, the viral integrase binds to the converted proviral DNA in the cytoplasma and forms the PIC. The complex is further processed by removing dinucleotide at each end of the DNA and producing new 3´-hydroxyl ends. In the next step, the pre-integration complex is transported from the cytoplasm of the cell to the nucleus through the nuclear pores. Inside the nucleus, the viral integrase binds to the chromosomal DNA of the host cell and mediates the integration of the viral DNA into the host DNA. In the last step, cellular DNA repair enzymes are repairing gaps in the DNA sequence, which might happened during the integration process (22). During viral replication, the integrated DNA is transcribed into RNA, some of which undergo RNA splicing to make messenger RNAs (mRNAs). These mRNAs are transported out of the

nucleus where they are translated into the regulatory proteins Tat, Rev, Nef, Vif, Vpu and Vpr. Tat accelerates the availability of viral RNA, Nef downregulates the presentation of the CD4 protein on the cell surface as well as enhances pathogenicity and Vif, Vpr, and Vpu influence the rate of virus particle production (12). However, Rev migrates back into the nucleus, binds to the complete unspliced RNA copies and allows them to leave the nucleus (23). Some of these full-length RNA copies function as new copies of the virus genome, while others function as mRNAs that are translated to produce the structural proteins Gag and Env. Gag catalysis the assembly of new virus particles. Therefore, Gag binds to copies of the unspliced virus RNA genome, together they move to the surface of the cell and assemble into a noninfectious immature virion. These virions are pushed out of the cells by a process called budding. Protease enzymes break up the polyprotein and allow the virus to assemble to its mature infectious form (24, 25).

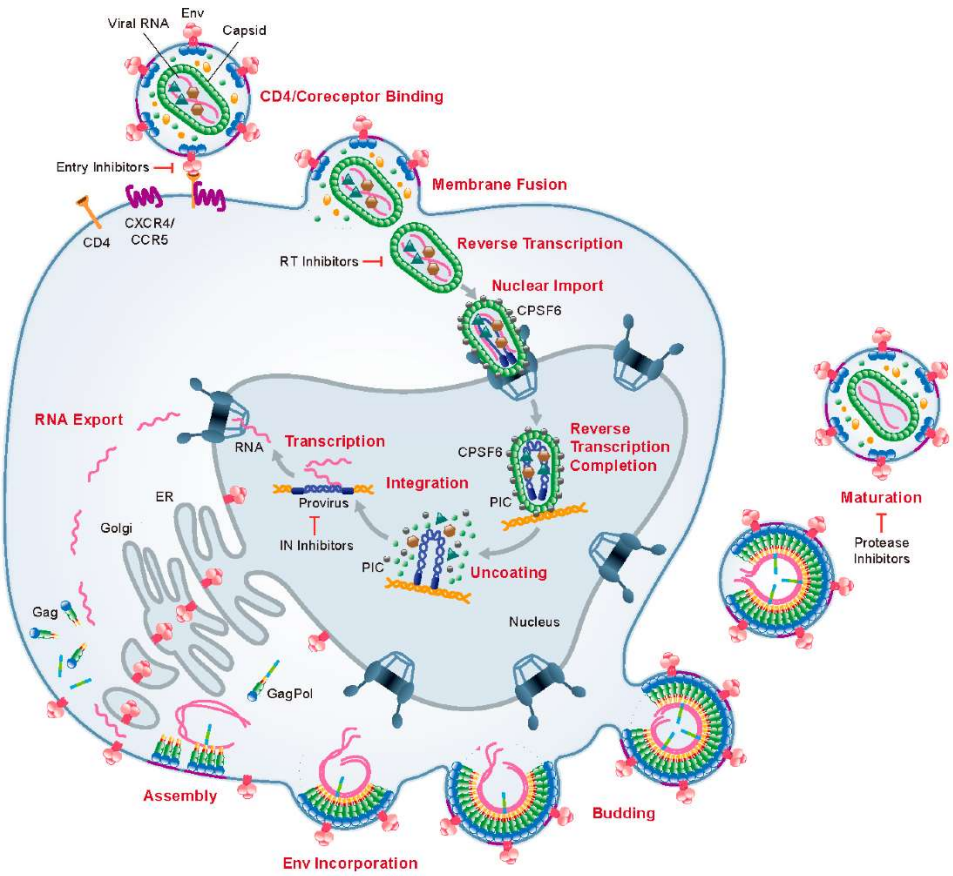

Figure 3: Individual steps of HIV-1 replication (figure from: (26)).

1.1.3 Transmission of HIV-1

The infection with HIV-1 can occur through different transmission routes. The most common transmission happens during unprotected sexual intercourse, vaginal or anal, with an HIV infected, viremic individual. The second common transmission results of sharing injection needles, which are contaminated with HIV infected blood from a viremic individual. The third common transmission route is a mother-to-child transmission during labor or breastfeeding. Transmission via contaminated blood during a transfusion is possible but mostly occurs in countries where blood is not routinely screened for HIV (27). The incidence of each transmission route can vary between countries and regions. In 2014, 40% of new HIV-1 diagnoses occurred in men who have sex with men (MSM) in Western Europe but only 28% in Central Europe. People who inject drugs (PWID) were 5% in Central Europe and 3% in Western Europe (17, 28).

1.1.4 Acute HIV-1 infection

The natural course of the HIV-1 infection is characterized by three different phases: (1) the acute infection, (2) the chronic infection and (3) the phase of AIDS-defining illnesses, which leads without antiretroviral therapy to death after a variable period of time. The acute infection of HIV-1 is defined by high levels of plasma HIV-1 RNA in the presence of a negative anti-HIV-1 ELISA and/or negative/indeterminate Western Blot and is part of the primary HIV-1 infection. A more detailed classification system of the early phases of the infection is defined by the Fiebig stages and characterized by the appearance of different viral markers and later on of antibodies in the blood (29, 30). The timeframe immediately after HIV-1 infection is the so-called eclipse phase and can last 7 to 21 days. During this time, the infection is established in local tissues at the exposure site and no level of HIV-1 RNA is detectable in the circulation (**Figure 4**). It follows a rapid expansion of HIV-1, first in gut-associated lymphoid tissue and then systemically, along with a drastic increase in plasma levels of viral RNA (30). During this ramp-up phase of acute HIV-1 infection, the infected individuals develop an intense inflammatory response characterized by high levels of cytokines and chemokines described as a cytokine storm (31). Throughout the acute phase, infected individuals often develop a “retroviral syndrome” which is associated with fever, rash, night sweats, severe fatigue, headache and/or diarrhea and occurs around two weeks after infection together with peak viremia. An early detection of the HIV-1 infection is important for early treatment start and a better disease outcome. Until recently, the 2nd

generation of enzyme immunoassay (EIA) was used to detect IgG antibodies against HIV-1 and was the standard screening method in the HIV diagnostic. However, the time between infection and reactivity (window period) is 25-35 days and limits the earliest time point of HIV-1 detection. (32). The 4th generation antigen-antibody combination EIA, which detects p24 antigen, HIV IgM and IgG shortens the window period and is now replacing the 2nd generation EIA (33).

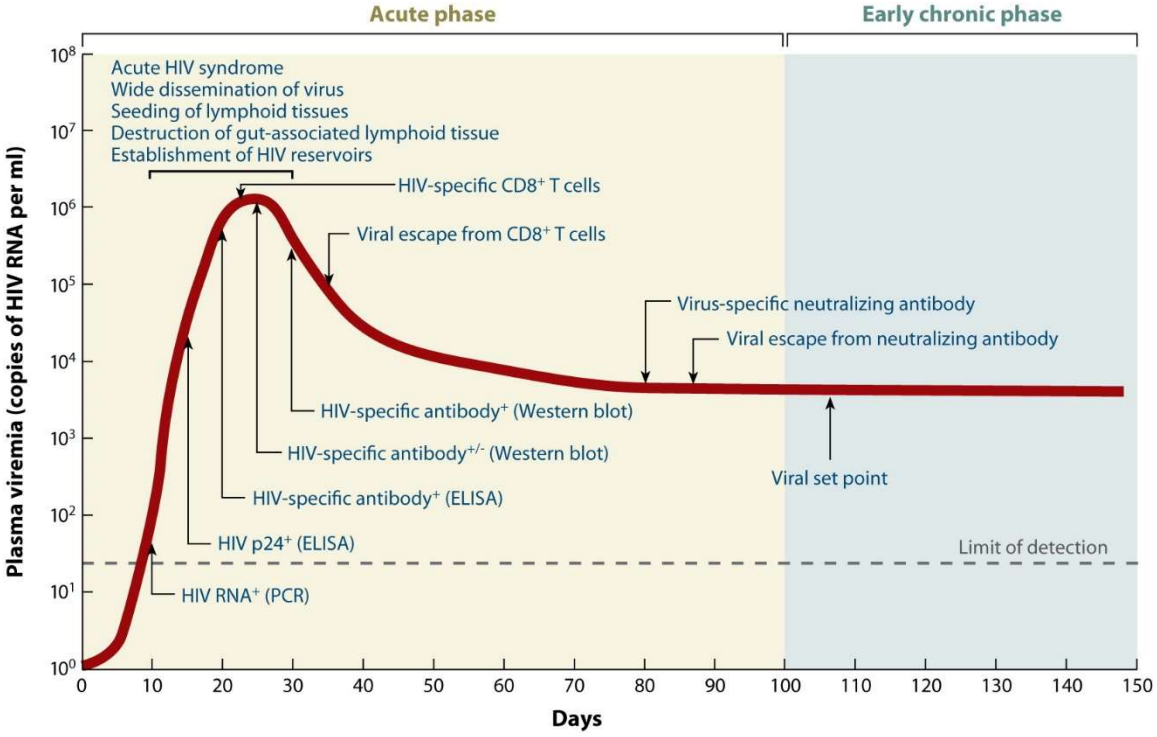

Figure 4: Kinetics of immunologic and virologic events during acute and early chronic phase (figure from: (34)).

In recent years several studies have investigated the biology behind the T cell response during acute infection. They have demonstrated that a strong CD8 T cell response early on can lower the viral set point and more importantly determine the long-term disease outcome (35, 36). HIV-1-specific CD8 T cells have the ability to kill HIV-infected cells and have been temporally associated with the initial decline of viremia (37, 38). CD4 T cells are the main targets for HIV-1 and during primary infection, a massive depletion of this cell type can be observed in almost all body compartments. The following impairment of CD4 helper function also affects CD8 T cell function and leads, among other factors, to an impairment of the CD8 T cells (39). However, studies have shown that HIV-1-specific CD4 T cell responses also contribute

to control of viral replication in HIV-1 infection and may predict disease outcome (40-42).

1.1.5 Chronic HIV-1 infection

HIV-1 infected individuals enter the chronic phase of the infection after the primary infection, which proceed mostly asymptomatic but with an ongoing increase in viremia and decrease in the CD4 T cell counts. In the early days of the HIV epidemic, HIV-1-infected individuals developed AIDS after a certain period of time and the life expectancy was approximately ten years. With the development of antiretroviral therapy (ART), the face of HIV and AIDS has changed from an almost always deadly infection to a manageable chronic disease (8). Indeed, individuals living with HIV have a comparable life expectancy to HIV negative individuals when treated early and efficaciously (43). The continuous intake of antiretroviral medication leads to full control of viremia and the partial restore of the immune system, which prevents the development of AIDS. However, an HIV-infected individual's health will never be fully restored and people who are on ART therapy might develop new health problems referred to as "non-AIDS morbidity". This includes cardiovascular disease, kidney disease, cancer, liver disease, or neurocognitive disease (44, 45) (Deeks, Lewin, and Havlir 2013; Freiberg et al. 2013). Direct toxicity of antiretroviral medication can contribute to these complications, although each new generation of antiretroviral drugs has been associated with less toxicity (46, 47). Nevertheless, until today HIV can still not be eradicated (48).

1.1.6 Antiretroviral Therapy

Over the last decades, several classes of antiretroviral medication were developed, which each inhibit distinct mechanisms during the HIV-1 replication cycle. Nowadays common ART regimens are using a combination of three antiretroviral agents against at least two distinct molecular targets to treat HIV+ individuals. This combination is needed because of the high diversity of HIV-1 and the quick emergence of drug-resistant HIV-1 strains within individuals. Overall, 30 individual or combined substances are being approved and used in current HIV-1 medication. They differ in their mode of action and can be divided into five different classes: (1) Nucleoside reverse transcriptase inhibitors (NRTIs), (2) Non-nucleoside reverse transcriptase inhibitors (NNRTIs), (3) Protease inhibitors (PIs), (4) Integrase inhibitors and (5) Entry inhibitors. A standard HIV-1 retroviral therapy consists of two drugs from the NRTI

class, which build the backbone of the therapy, and these are combined with one drug from the other classes (**Figure 5**) (8).

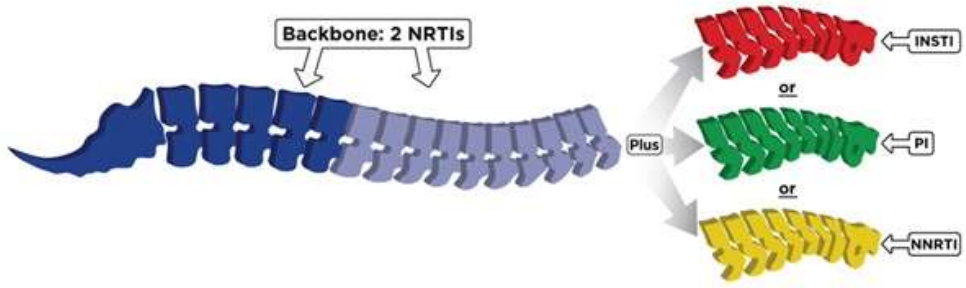

Figure 5: Standard HIV-1 retroviral therapy backbone (figure from: (49)).

1.1.6.1 Nucleoside reverse transcriptase inhibitor (NRTI)

When the U.S. Food and Drug Administration (FDA) approved with Zidovudine (AZT) the first antiretroviral drug in 1987, it belonged to the class of nucleoside reverse transcriptase inhibitors (NRTIs) (50). This class targets the viral reverse transcriptase and inhibits the conversation of viral RNA into DNA. NRTIs are nucleoside analogs, which compete with the natural nucleosides during reverse transcription. Because of a minor modification in the ribose molecule of the NRTIs, the incorporation of the nucleoside analogs causes a destabilization of the double-stranded DNA by preventing the build of phosphodiester bridges. This leads to a stop of the DNA synthesis by the reverse transcription. Nucleoside analogs are pro-drugs, which needs the host cell for entry and phosphorylation by cellular kinases (51-54). The problem of long-term treatment with NRTIs, as with all antiretroviral drugs, is a possible decreased drug susceptibility of the HIV-strain caused by mutation. The HIV resistance of the strain can be achieved by two different mechanisms, referred to as discrimination or extinction pathways. In discrimination pathways, mutation cause changes in the primary structure of the reverse transcription, which increases the selective ability to incorporate the correct natural nucleosides. On the contrary, the extinction pathway causes an alteration in the primary structure, which leads to a functional change of the reverse transcriptase. It facilitates removing the chain terminators NRTI-triphosphate from the 3´ end of the DNA chain after it has been incorporated (55-59).

So far there are nine FDA approved NRTIs available to treat HIV-infected individuals: abacavir (ABC), didanosine (ddI), lamivudine (3TC), stavudine (d4T), zalcitabine

(ddC), zidovudine (AZT), tenofovir disoproxil fumarate (TDF), emtricitabine (FTC) and tenofovir alafenamide (TAF). It has been shown in the past that there are long-term side effects of NRTIs including lactate acidosis, myelotoxicity, polyneuropathy, or pancreatitis but also with metabolic disorders, in particular lipoatrophy. In addition, side effects were also observed in mitochondria, which require nucleosides for proper function. It has been shown that the incorporation of the nucleoside analogs by the mitochondrial polymerase gamma leads to a mitochondrial degeneration and affects the metabolism of mitochondria (60-62).

1.1.6.2 Non-nucleoside reverse transcriptase inhibitor (NNRTI)

The second class of antiretroviral drugs was described in 1990 and function as noncompetitive inhibitors. They are small hydrophobic chemical compounds, which bind directly to the reverse transcriptase in a pocket far from the active site. These results in a change in the residues' structural conformation in the active site, which again reduces its activity to catalyze the DNA synthesis (63-67). NNRTIs are characterized by their long half-life and low level of resistance barrier. These results in a fast appearing of resistance, mainly from a mutation in the inhibitor binding site (68). Furthermore, resistance against NNRTI usually generates additional cross-resistance to other NNRTIs (69).

Up to now, there are five NNRTIs available. The first generation includes nevirapine (NVP), delavirdine (DLV), and efavirenz (EFV) which were introduced to the market in 1996 (70). The second generation represents etravirine (ETV) as well as Rilpivirine (RLP), which were approved for HIV-1 treatment in 2008 and 2011, respectively. Overall, NNRTIs are well tolerated and show good efficacy combined with NRTIs (71-73). However, EFV is linked to neuropsychiatric toxicity and mitochondrial alterations (74).

1.1.6.3 Protease inhibitor (PI)

Protease inhibitors (PIs) are small chemical molecules, which function as competitive inhibitors and prevent the viral protease from cutting the gag-pol polyprotein into functional subunits. Therefore, no infectious virus particles can be produced. Because PIs are competitive inhibitors, they mimic the gag-pol polyprotein, bind to the active site of the protease and block the access for the natural viral gag-pol polyprotein (75, 76).

There are several PIs approved for HIV-1 treatment: amprenavir (APV), atazanavir (ATV), darunavir (DRV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV), saquinavir, and tipranavir (TPV). Overall boosters are needed to increase the available plasma concentration in the blood. So far, two drugs are used as boosters – RTV or cobicistat. RTV blocks the function of the hepatic isoenzyme CYP3A4, which increases the plasma concentration of all PIs (77). Escape mutations against PIs primarily occur in the active site of the viral protease, which affects the binding capacity of the drugs but not of the gag-pol polyprotein (78).

1.1.6.4 Integrase strand transfer inhibitors (INSTIs)

The group of INSTI is the most recently discovered class of antiretroviral drugs, which the first drug was approved in 2007 in the USA and Europe. Nowadays, INSTIs are very commonly used and are part of first-line HIV antiretroviral regimens. INSTIs function as competitive inhibitors, which bind to the active site, block the HIV-1 enzyme integrase's specific function to combine viral and cellular DNA, and prevent the important incorporation of viral DNA into the host genome (79).

The first approved INSTI was raltegravir (RAL), followed by the quinolone derived INSTI elvitegravir (EVG) and the second-generation of INSTI dolutegravir (DTG). In 2018, the newest INSTI bictegravir was approved by the FDA to treat HIV-1 infected individuals (80-82). So far, treatments with INSTIs are generally well tolerated, however negative effects on the metabolism of CD4 T cells have been reported recently (82).

1.1.6.5 Entry inhibitors

This class of inhibitors interferes and prevent the receptor-mediated entry of HIV-1 into the cell. Because of the different phases during the entry process, there are two different classes of entry inhibitors: co-receptor antagonists and fusion inhibitors. The only approved co-receptor antagonist for treating HIV-infected individuals is maraviroc, which is a CCR5 antagonist. It binds allosterically to the co-receptor CCR5 on the cell surface, induces conformational change and prevents the binding of viral gp120 to CCR5. HIV-1 strains, which use the co-receptor CXCR4, would not be blocked (83, 84).

An FDA-approved fusion inhibitor is enfuvirtide. It is a peptide chain that mimics the heptad repeat (HR1) structure of HIV-1 gp41. During the fusion process, the HR1 structure binds to the heptad repeat region (HR2) on the cell surface and facilitates the

fusion of viral envelope and cell membrane. Binding of the peptide chain into the HR1 region prevents binding of HR2 and blocks the fusion (85).

1.2 Immune system

There are two arms of the human immune system: the innate immune system and the adaptive immune system. The innate is very quick in contrast to the adaptive immunity, where immune responses can develop after several days.

1.2.1 Innate immune system

The innate immune system is characterized as the first line of defense against invading microbes. The response of this arm of the human immune system occurs rather very fast, right after exposure to foreign antigens. It represents the oldest defense mechanism and certain components, such as antimicrobial proteins secreted on the mucosal surfaces, are functional at all times even before encounter with infectious agents, specific in the skin and epithelial barriers in the gut and respiratory tract (86). In case microbes cross the initial barriers, they next encounter a major component of innate immunity known as the complement system. This system represents a collection of different soluble proteins (complements), which are present in the blood or other body fluids. In the absence of infection, the different complement proteins patrol throughout the body in their inactive form. As soon as they recognize an invading pathogen, they can get activated by three different pathways: the antibody-triggered pathway, the alternative pathway, which can be activated by the presence of the microbe alone or the lectin pathway, which can be activated by lectin-type proteins binding to the surface of pathogen. The outcome is the same for all three complement activation pathways and results in killing the invading pathogen. This can be accomplished for example, through the recruitment of phagocytic cells to the site of infection (**Figure 6**) (87).

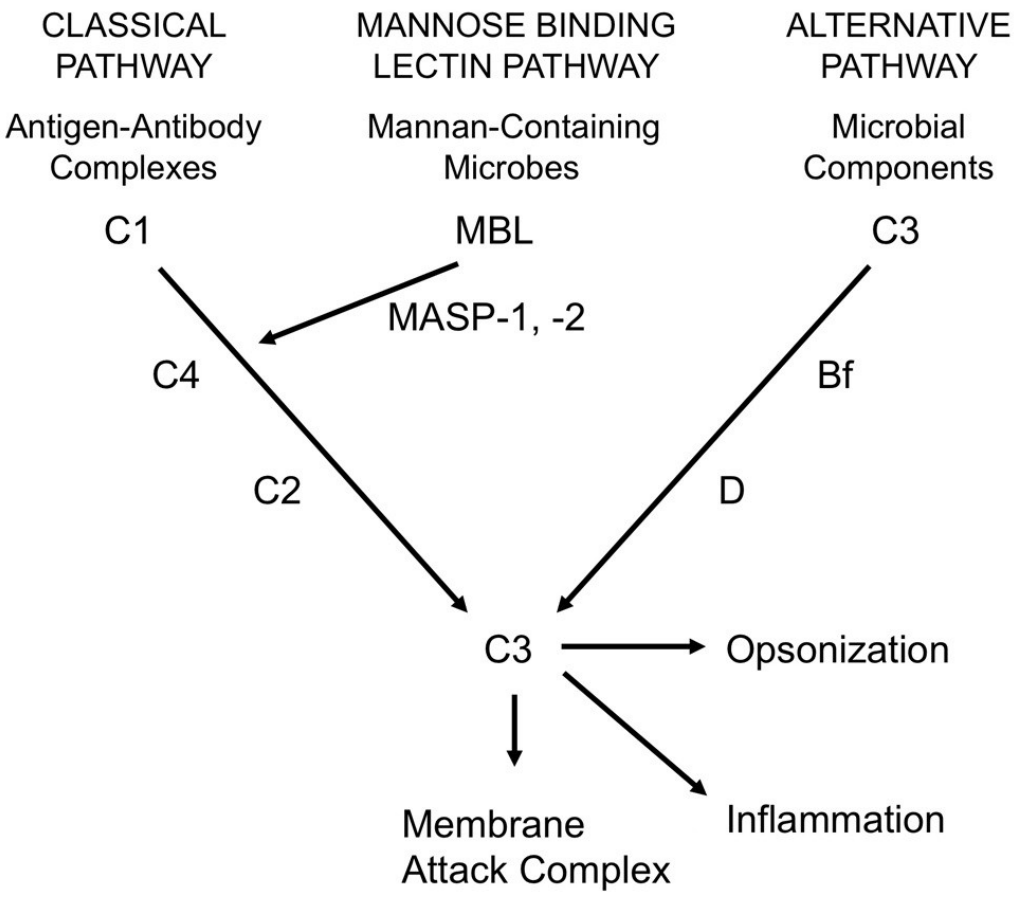

Figure 6: Complement activation by three different pathways (figure adapted from: (88)).

Another line of defense is the recognition of the pathogens by the resident phagocytic cells, which can be divided into three major classes: macrophages and monocytes, granulocytes (neutrophils, eosinophils, basophils) as well as dendritic cells and natural killer cells. Because macrophages are present in the submucosal tissues, they are mostly the first cells to encounter the invading pathogens. Not long after the first contact with the infectious agent, a large number of neutrophils are recruited to the sites of infection. Both cell types recognize pathogens through cell-surface receptors than can discriminate between the surface molecules of pathogens and those of the host. The main recognition receptors on the surface of these innate immune system cells are toll-like-receptors (TRLs). In total, there are ten known human variants, which are all recognizing different molecules only expressed on microbes but not mammalian cells. The activated response of the immune cell depends on the specific TLR and can be vary between the expression of inflammatory cytokines or costimulatory molecules. The presented molecule structures on the surface of the microbes, which are recognized by the TLRs, are the pathogen-associated molecular patterns (PAMPS).

These are different microbial products, which are essential for the survival of the pathogen (89, 90).

Besides eliminating invading pathogens, the innate immune system is essential for the stimulation response of the adaptive immune system. Naïve lymphocytes require priming by antigen presentation of macrophages or dendritic cells. This priming ensures that the following response of lymphocytes is antigen-specific. Moreover, the innate immune system can provide additional stimuli in the form of either costimulators, cytokines, or complement breakdown products (88).

1.2.2 Cells of the innate immune system

1.2.2.1 Macrophages

Macrophages are the major phagocyte population located in connective tissue (like the submucosal layer of the gastrointestinal tract), which are able to take up invading microbes by phagocytosis. Therefore, they express different cell-surface receptors that bind to pathogens and induce phagocytosis of the intruder (91).

1.2.2.2 Granulocytes

Granulocytes are a major family of phagocytes, which include neutrophils, eosinophils and basophils. They can recognize, ingest and destroy many pathogens without the help of the adaptive immune system. Neutrophils are capable of phagocytosing pathogens and they are one of the first types of innate cells to migrate toward sites of inflammation (92). Eosinophils are mostly responsible for fighting against invading parasites. After activation, they release their granular contents, including enzymes, growth factors and a variety of cytokines (93).

1.2.2.3 Dendritic cells

Dendritic cells have been found in all tissues throughout the body and especially in the peripheral lymphoid organs. One of the main role of dendritic cells is to function as bridge between innate und adaptive immune response. They can, similar to macrophages, ingest, break down pathogens and afterwards generate peptide antigens, which can be presented to T cells. A special subset of dendritic cells, the plasmacytoid dendritic cells, can also produce antiviral interferons (94, 95).

1.2.2.4 Natural killer cells (NK cells)

NK cells are large cells, which are able to kill stressed, infected or malignant cells by releasing cytotoxic granules containing granzymes and perforin (96). The decision of

a NK cell to kill another cell is determined by a number of activating or inhibitory signals that are generated through detection of the ligands that are present on the surface of that cell. NK cells are able to recognize changes in cell surface glycoprotein composition caused by infection or malignant transformation. This involves, for example, the downregulation of major histocompatibility complex (MHC) molecules caused by the invading pathogens. NK cells cannot bind to the MHC molecules, recognize this imbalance of signals, become activated and kill the infected cell (97).

1.2.3 Adaptive immune system

The adaptive immune response to an unknown pathogen normally sets in a few days after the innate immune response, but it targets the pathogen more accurately. The adaptive immune system consists of T and B-lymphocytes and can be found circulating through the lymph and distributed all over the other organs and tissues of the body. The antigen receptors on the surface of B cells and the antigen-specific receptors on T cells are responsible for recognizing the foreign antigen and the following response of the immune cells. In comparison to the innate immune system, after antigen presentation by antigen-presenting cells (APC) to naïve lymphocytes, they can develop either to effector- or memory cells. Effector cells can provide immediate action, where memory cells remember the foreign antigen and provide long-lasting immunity, which will later result in a much faster immune response.

1.2.4 Cells of the adaptive immune system

1.2.4.1 B lymphocytes (B cells)

B cells represent the humoral response of the adaptive immune system and the principal function of this cell type is the production of antigen-specific immunoglobulin (Ig), called antibodies. B cells arise from progenitors derived from hematopoietic stem cells (HSCs) and can be classified in B1 and B2 lymphocytes. B1 lymphocytes arise from B1 progenitors in the fetal liver, while B2 lymphocytes develop from transitional 2 B cells that originate from bone marrow precursors. B1 lymphocytes are located in the pleural as well as peritoneal cavities and are characterized by T cell independent responses. B2 lymphocytes are located in the secondary lymphoid organs of the immune system and consist of B cells which are located in the marginal zone (MZ) or the follicular zone (FO) of the secondary lymphoid organs (**Figure 7**). FO B cells are the most numerous B cell lineages, their response is T cell dependent and are primarily responsible for the generation of long-lasting, high-affinity IgG antibodies (98).

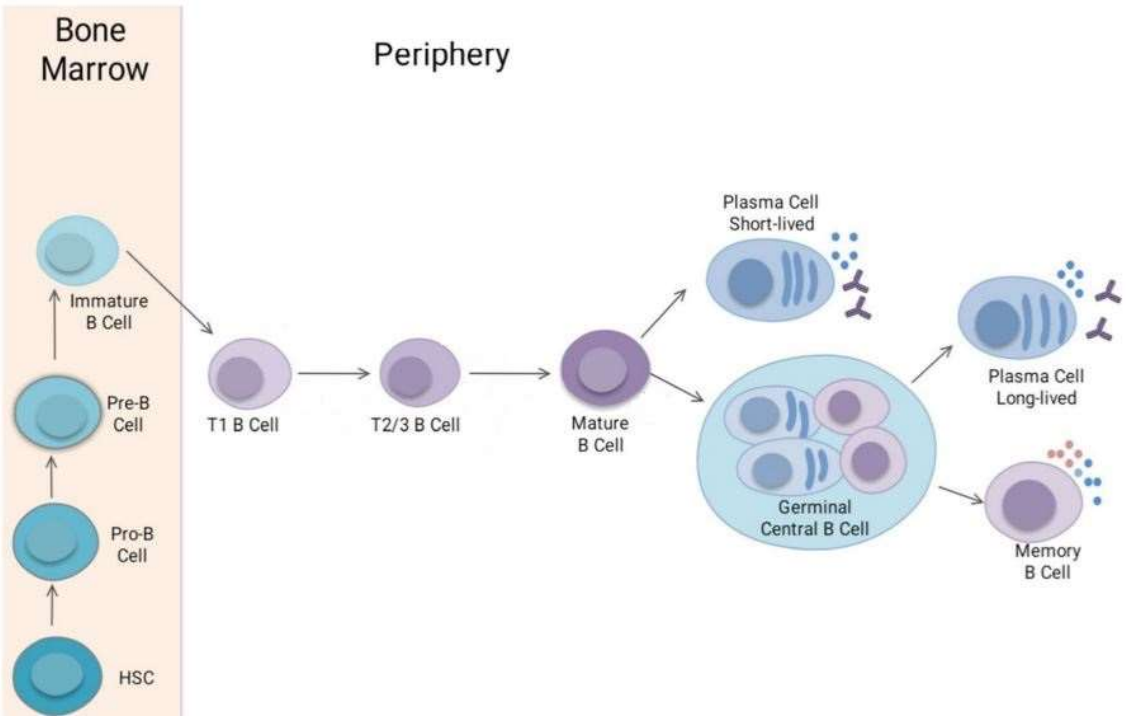

Figure 7: B cell differentiation pathways (figure adapted from: (99)).

Antibodies are glycosylated protein molecules, which can be located on the surface of B cells (B cell receptors, BCR) or are secreted into the extracellular space, where they can bind target antigens and kill the invading pathogen. In total, there are five known classes of antibodies IgM, IgD, IgG, IgA and IgE, which differ in their biological properties, functional locations and ability to respond to different antigens. In addition, there are four subclasses of IgG antibodies (IgG1, IgG2, IgG3, and IgG4). They are all composed of two heavy chains (IgH) and two light chains (IgL), representing the Fab and the Fc fragment. Through the development process of B cells there is a rearrangement (V (D) J) of the heavy- and light-chain gene segments, which secure the production of a massive selection of BCRs and therefore, bind many different antigens. The Fc fragment consists of two heavy chains, not participating in the antigen binding but is important for the antibody's effector functions of the antibody. Instead, the antigen-binding fragment, Fab, is composed of one variable domain from the heavy and one from the light chain. These variable domains contain the complementarity determining regions (CDRs) with the most sequence variations and determine the important antibody specificity (100).

Through the binding of antigens to the BCR of naïve B cells, they undergo as immunoglobulin class switching and proliferate via clonal expansion to become either

a plasma or a memory B-cell. Plasma B-cells secrete low-affinity soluble antibodies (IgM and IgD), binding the same antigen and eliminating the pathogen (**Figure 7**). However, Memory B-cells remain in the body for long-term immunity and are able to secrete higher-affinity IgG or IgA antibodies (101).

1.2.4.2 T lymphocytes (T cells)

Similar to B lymphocytes, T cells arise from progenitors derived from hematopoietic stem cells in the bone marrow, where they eventually migrate to the thymus for development. In the thymus, progenitor cells are firstly located in a network of epithelia, known as thymic stroma, where they receive developing signals from stromal cells. Developing progenitor cells undergo a series of maturation steps, which can be defined by the expression of the T cell receptor (TCR) and by changes in the expression of cell surface markers. In one of the first maturation steps, progenitor cells lack the expression of CD3 as well as of the co-receptors CD4 and CD8. These so-called double negative (DN) cells derived in the next step to two distinct lineages of T cells with different types of T cell receptor chains. DN cells pass through different maturation stages, where they proliferate, undergo rearrangement of their TCR chains and eventually upregulate CD4 and CD8 expression (double positive cells, DP). DP cells then migrate to the cortex of the thymus, where they finally differentiate into single positive CD4 or CD8 T cells. Interestingly, only a very small percentage of progenitor cells leave the thymus as mature T cells. T cells have to interact constantly with self-peptides in the cortex, which are displayed on by specialized host-cell glycoproteins on cortical epithelial cells - MHC I or MHC II. This interaction with the self peptide-MHC complexes ensure the survival of the immature T cells and also will determine whether the DP cells differentiate in CD4 or CD8 positive T cells. DP cells that interact in a weaker affinity die by apoptosis to guarantee that matured T cells do not react to self-antigen. Mature naïve CD4 or CD8 T cells exit the thymus into the blood stream or secondary lymphoid tissues (102, 103). There they get in contact with APCs which presents a foreign antigen, whereupon naïve T cells become activated, proliferate, and differentiate into effector cells. The recognition of antigens just can happen, if those are displayed in a specific way on the surface of the host cells. Antigen-presenting cells (APCs) mainly incorporate foreign proteins or peptides by endocytosis, which they then get delivered to the cell surface of the APC by MHC molecules. There are two classes of MHC molecules, both different in their structure and expression pattern in the tissues. MHC class I molecules present peptides from pathogens, commonly viruses,

to CD8 T cells, whereas CD4 T cells recognize antigens presented on MHC class II molecules. After priming through APCs, activated CD4 and CD8 T cells recognize antigens presented by their appropriate MHC molecule (104).

1.2.4.2.1 CD4 T cells

T-lymphocytes that express the CD4 glycoprotein are very important and exert various functions for the adaptive immune system. Their main functions help to coordinate, suppress or regulate immune responses and therefore known as T-helper cells. Nevertheless, is has been shown that CD4 T cells could themselves exhibit cytotoxic activity and kill infected cells. In contrast to CD8 T cells, CD4 T cells are able to differentiate into many different subsets: Th1, Th2, Th9, Th17, Th22, Treg (regulatory), T_{FH} (follicular helper) and cytolytic CD4 T cells. The differentiation into each subset is driven by different cytokine contents of their surroundings (**Figure 8**). Every CD4 T cell subset is characterized by a slightly different function and expression of their secretory cytokines. Their effector function defines the Th1 and Th2 subsets of CD4 T cells. Th1 CD4 T cells are secreting IFNγ, TNFα, and IL-2 which both upregulates Th1 differentiation and downregulates Th2 differentiation at the same time. Through the secreted cytokines, Th1 subset especially mediates immune responses against intracellular pathogens (105). In contrast, Th2 CD4 T cells differentiate mainly after infection with extracellular parasites, including helminths. They express IL-4 and IL-13 as their key cytokines, which promote the production of specific IgE antibodies by B cells. On the contrary, both CD4 T cells subsets are known to be involved in the pathogenesis of autoimmune diseases like Crohn's disease (Th1), or chronic inflammation associated with allergic diseases (Th2) (106, 107). Despite the effector function of CD4 T cells subsets, T regulatory CD4 T cells (Tregs) are very important in regulating immune responses and maintaining self-tolerance. Tregs can suppress immune responses through different mechanisms like binding to APCs to prevent other T cell interaction or through their production of cytokines, including TGF-β, IL-10, and IL-35, which negatively affect the effector function of other immune cells. An altered Treg subset or disturbed Treg signaling can cause autoimmune diseases like systemic lupus erythematosus (108).

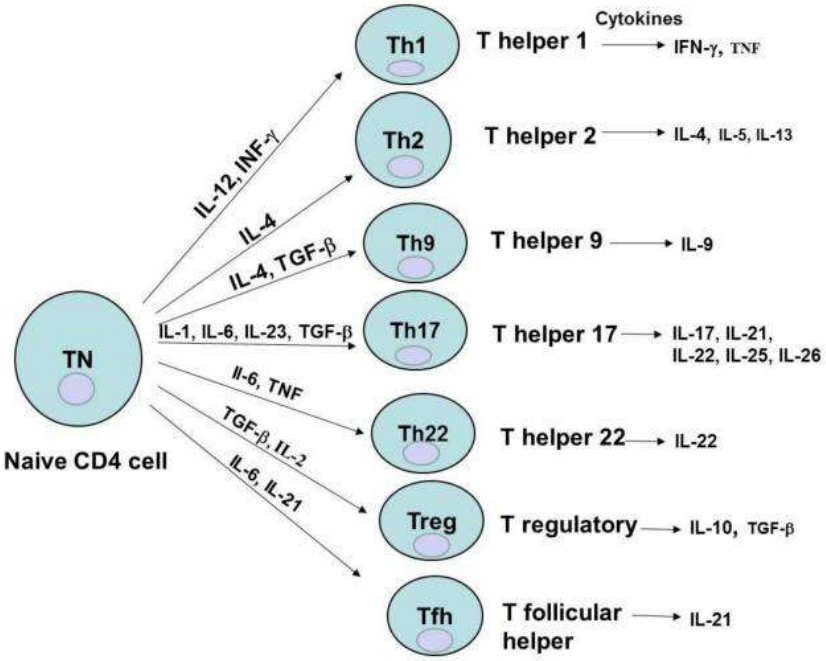

Figure 8: The different CD4 T cell subsets (figure from: (109)).

Although these CD4 T effector cells are very effective, they are also relatively short-lived. Memory CD4 T cells live much longer and are part of the immune memory. They are mostly located in secondary lymphoid organs (central memory T cells, T_{CM}) or in the recently infected tissue (effector memory T cells, T_{EM}). When the immune system come across the same pathogen again, the memory T cells proliferate an immune response occurs much faster (109, 110). The role of CD4 T cells in HIV-infection is thematized in **1.2.5**.

1.2.4.2.2 T follicular helper cells

T follicular helper cells (T_{FH}) is a special subset within the CD4 T cells and play a crucial role in delivering important survival and differentiation signals to B cells as well as supporting antibody affinity maturation. T_{FH} cells are mainly characterized by their ability to co-express the chemokine receptor CXCR5 together with the surface molecule PD-1 and by their secretion of the cytokine IL-21. The expression of CXCR5 enables the ability to migrate into the B cell follicle within the secondary lymphoid organs and relocate inside the germinal centers. CD8 T cells or other CD4 T cells, which not express CXCR5, cannot enter the follicle and this colocalization of T_{FH} cells with B cells is critical for interaction and supporting of B-cell function. The master regulators Bcl6 and c-Maf primarily drive the differentiation of a naïve CD4 T cell into a T_{FH} cell, which expresses CXCR5. Both transcription factors cooperate in the

induction of T_{FH} key markers CXCR5, PD-1 and ICOS. Furthermore, c-Maf is an important factor of cytokine production in the later stages of T_{FH} development and induces IL-21 expression (111-113). In addition to it, the inducible T cell co-stimulator (ICOS) on the surface of T_{FH} cells can interact with the ligand of ICOS on the surface of B cells and is important for progression and maintenance of T_{FH} cells in the GC. T_{FH} cells express different surface molecules to interact with B cells and secret IL-21 as a key cytokine, involved in numerous T_{FH} cell functions. CD40 ligand (CD40L) as a surface molecule engages with CD40 receptor on the surface of B-cells and can promote B-cell proliferation and differentiation. IL-21 as the key cytokine expressed by T_{FH} cells can induce B cell proliferation and more importantly differentiation into Ig-producing plasma cells (114-117). The special problematic of T_{FH} cells in HIV-infection is thematized in **1.2.5**.

1.2.4.2.3 Cytolytic CD4 T cells

Despite the traditional role of CD4 T cells to help B cells, it was early on noticed that CD4 T cells could themselves exhibit cytotoxic activity (118). This function, normally key purpose of CD8 T cells, is characterized by killing other cells through the cytolytic molecules granzyme and perforin in humans, or through Fas Ligand-mediated killing shown in mice (119). Differentiation in cytolytic CD4 T cells (CD4 CTL) is still under investigation, but different studies have revealed different cellular origins. It is possible that CD4 CTL can develop from naïve CD4 T cells, but also from Th1, Th2, Th17 or Tregs effector subsets. Nevertheless, the development from Th1-like CD4 T cells represents the majority of CD4 CTL. Primarily involved in the development of CD4 CTL are the T-box brain protein 2 (EOMES) and the T-box transcription factor TBX21 (T-bet). These proteins bind to a specific sequence in the DNA (T-box). This activates the expression of transcriptions factors, promoting the expression of IFNγ and cytotoxic granules (**Figure 9**). Interestingly, EOMES is highly expressed in CD8 T-cells, where regular Th1 CD4 T-cells have a lower amount of EMOES and higher amount of T-bet (114, 120). So far exclusive marker to identify CD4 CTL have not been described yet. Similar to other cytotoxic cells, CD8 T cells or NK cells, CD4 CTL express lysosome-associated membrane glycoproteins (LAMP) on their surface, especially LAMP-1 (CD107a). These are proteins localized in lysosomes, which are a host of different granzymes and perforin. Upon activation, lysosomes are recruited to the cell surface, where they fuse with the plasma membrane and secrete their content via exocytosis in the intracellular space. Through the process of exocytosis, CD107a will be presented

for a short time on the surface of the cell, until it will be downregulated again (121). Furthermore, the expression of granzyme A, B and K, as well as perforin and IFNγ of CD4 T cells, can also be used in humans to identify CD4 CTL (42).

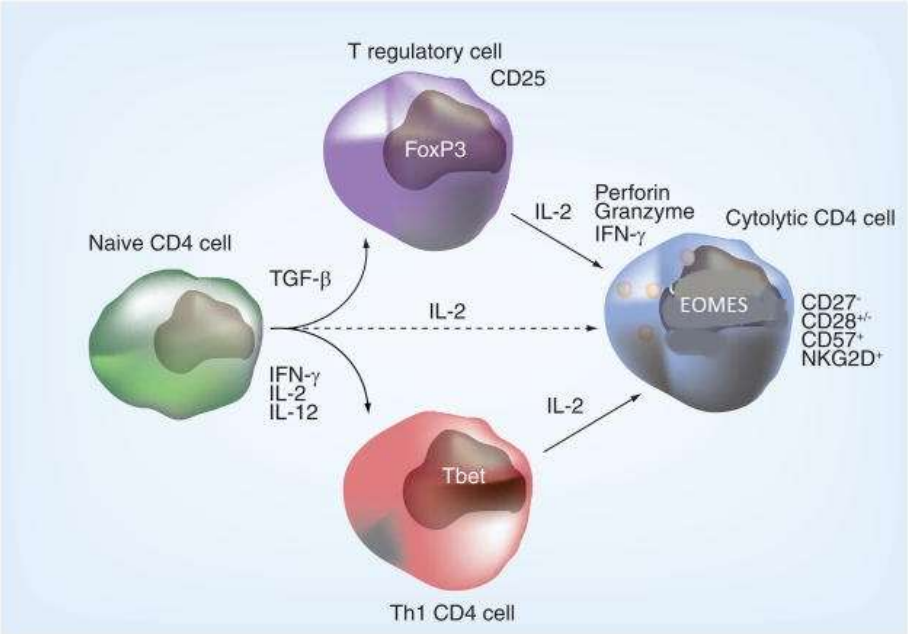

Figure 9: Cytolytic CD4 T cell lineage (figure adapted from: (42)).

Since CD4 CTL were described, several studies showed the importance of CD4 CTLs to contain viral replication in Epstein-Barr virus, cytomegalovirus or Influenza A virus infection (122, 123). In HIV infection it has been shown that HIV-specific cytolytic CD4 T-cells emerge early during acute HIV infection and are associated with early viral control as well as better disease outcome and they phenotypically, functionally and transcriptionally distinct from other CD4 T cell subsets (42, 124, 125).

1.2.4.3 CD8 T cells

T-lymphocytes that express the CD8 glycoprotein are very important due to their cytolytic capacity and their role in the defense against intracellular pathogens like viruses or bacteria and tumor surveillance. Despite CD4 T cells, which mainly focused on the regulation of the immune response, CD8 T cells aim to kill infected cells after peptide presentation from APCs. Therefore, they can use three distinct pathways to control infection or tumor surveillance. The first way is described by the expression of cytokines, particularly TNFα and IFNγ cytokines, which are mainly characterized in their anti-tumor and anti-microbial effects (**Figure 10**). The second, and most common mechanism, involves the expression of cytolytic molecules granzyme A, B or K as well

as perforin. They are usually stored in cytotoxic granules inside CD8 T cells and get secreted in the intracellular space (so-called immune- or cytolytic synapse) upon activation. Through the binding of the CD8 T cell to the peptide of the surface of the target cell, it is ensured that the cytotoxic granules are released only in the direction of the infected or malignant cell. Once in the cytolytic synapse, perforin forms a pore in the membrane of the target cells and allows granzymes to enter the infected or malignant cell through this pore. Granzymes are serine proteases that cleave proteins inside the target cell, shutting down viral proteins' production and ultimately resulting in apoptosis of the infected or malignant cell (104, 126). FasL-Fas interaction induces the third mechanism of killing. CD8 T cells that got activated express FasL on their surface and can bind the Fas receptor on the surface of target cells. This interaction between ligand and receptor initiates downstream a caspase cascade (firstly caspase 8 followed by caspase 3) in the target cell, which results in apoptosis of the target cells. Interestingly, CD8 T cells can as well express the Fas receptor, what represents a sort of regulation mechanism how CD8 T cells can kill each other, called fratricide (104). The role of CD4 T cells in HIV-infection is thematized in **1.2.5**.

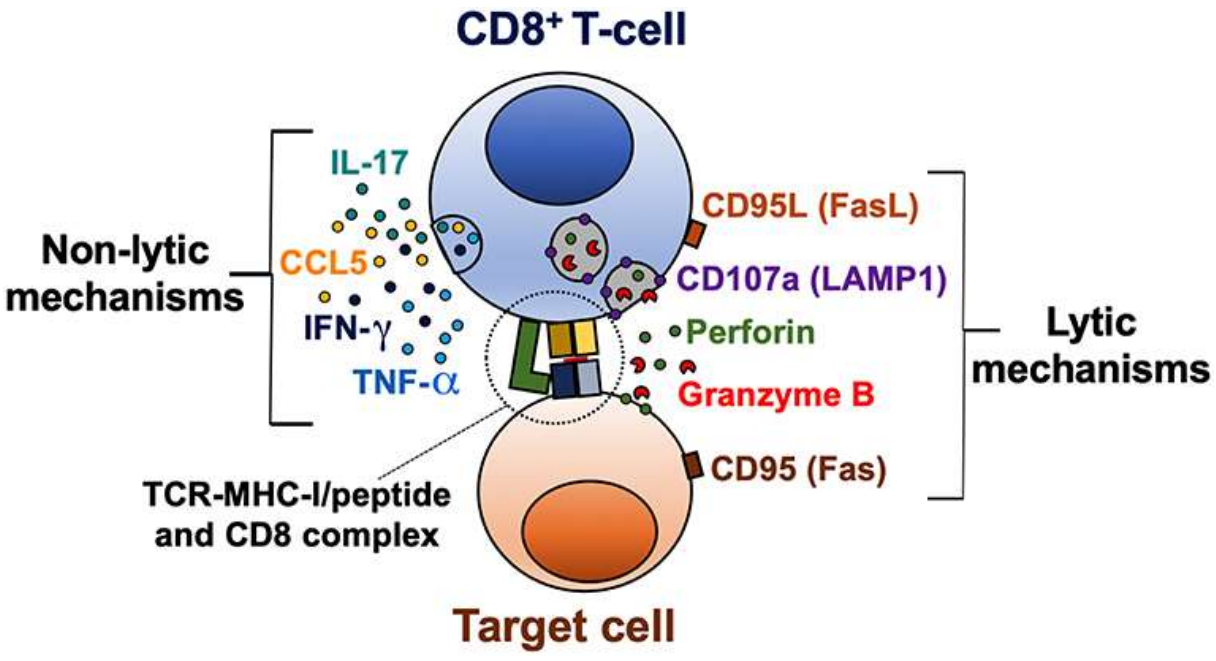

Figure 10: Lytic and non-lytic effector mechanisms of CD8 T cells (figure from: (127)).

In many viral infections, CD8 T cells are important for mediating clearance of the viral infection and the capability of memory CD8 T cells to provide protection against

secondary infections (128). CD8 T cell response during HIV-infection is thematized in the following.

1.2.5 HIV-1 and immune system

Changes in the immune response during HIV-infection can be described for different phases of the infection (**Figure 11**). During the acute HIV-1 infection individuals develop typical symptoms as sore throat, fever, swollen glands or a rash. These symptoms are caused by the rapid immune activation shortly after infection and characterized by a massive release of different cytokines, including IFNγ, TNFα, IL-6, IL-10, IL-15 (31). During HIV-1 infection, CD4 T cells become the major target of the virus. Shortly after infection they are being massively depleted and especially follicular helper CD4 T cells get infected with HIV-1 in large numbers (29, 129). It has been reported, that T_{FH} are the major cell compartment contributing to viral persistence of a long-lasting viral reservoir (130, 131). CD8 T cells, on the other hand, play an important role in controlling the virus during the acute phase of the infection. HIV infection induces a robust and durable HIV-specific CD8 T cell response. The frequency of activated CD8 T cells increases up to 50% and studies have shown that the quality of the CD8 T cell functional response during early HIV-1 infection can determine long-term disease outcome. HIV-specific CD8 T cell responses can stabilize the viral set point and control the viral replication to a certain degree (36). As the infection progresses, HIV-1 can evade the influence of CD8 T cell-mediated immune control. Possible causes are escape mutations within the targeted CD8 T cell epitopes and chronic immune activation (132-134). Based on clinical observations and blood analyses from chronically HIV-infected individuals, it has been shown that CD8 as well as CD4 T cells undergo a chronic immune activation, which is associated with morbidity and mortality (135). With infection progression, studies have shown a strong increase of the late-activation marker (CD38 and HLA-DR), proliferation marker (KI-67) and exhaustion marker (PD-1, CTLA-4) on both CD4 and CD8 T cells (136). A high number of activated and exhausted CD8 T cells were associated with CD4 T cell depletion, loss of function in CD8 and CD4 T cells, increased HIV-1 viral load and development of AIDS (137, 138). Nevertheless, studies have shown that CD4 T cells also contribute to the control of infection progression. HIV-specific cytolytic CD4 T cells emerge early during acute HIV infection and are associated with early viral control (124, 125). In addition, rhesus macaques in which CD4 T cells were depleted prior to infection

showed no post-peak decline of viremia and rapid disease progression suggesting that CD4 T cells are essential in establishing control of virus replication (139).

The development of potent ART strategies has significantly changed the long-term progression of the infection. While the majority of infected people develop AIDS without therapy, ART-treated individuals can control the viral load to an undetectable level. However, despite a partial improved function, HIV-specific CD8 T cells on ART therapy show still signs of overall dysfunction in comparison to HIV-uninfected individuals (140). In contrast, there is a small percentage of infected people who demonstrate to control virus replication without ART: elite controllers. These individuals can maintain low or undetectable viral loads without any ART. Previous studies have shown that HIV-specific CD8 T cells from elite controllers have enhanced cytotoxic function compared to progressor patients (141). Nonetheless, despite the effective controlling of viral load, ART cannot fully eradicate HIV infection and HIV persists in a small, dormant reservoir (thematized in **1.3**).

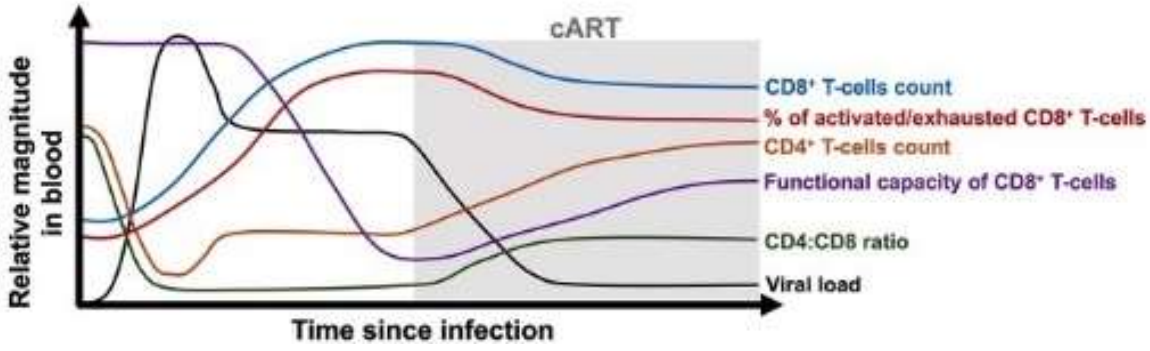

Figure 11: Model of the dynamic of viral load as well as CD4 and CD8 T cells (figure from: (127)).

Approximately 50% of HIV-infected individuals develop neutralizing antibodies during their progression of the infection (142). Due to high variations in the genotype of HIV-1, antibodies against this virus need to be able to act on the breadth of these genetic variations (143). These antibodies are known as broadly neutralizing antibodies (bnAbs). HIV-1 infected individuals develop type-specific cross-neutralizing antibodies, but only a few of these individuals manage to develop very potent bnAbs (144). Some of these potent bnAbs have been isolated and shown promising results in animal models, as well as in humans, and have shown modest and transient suppression of viremia (145, 146).

1.3 HIV-1 reservoir

Although ART treatment can control viral load in the blood of HIV-infected individuals, therapy fails to eradicate HIV and an interruption of ART leads to a rebound in viremia (147). It is believed that HIV-1 persists in a small pool of latently infected CD4 T cells in different lymphatic compartments throughout the body, especially in the lymph nodes and the gastrointestinal tract (148, 149). It has been demonstrated that the lymph node harbors high levels of infected CD4 T cells and that in particular T_{FH} cells within the lymphoid B cell follicle are massively infected with HIV-1 (150, 151). The B cell follicle structure promotes the possibility for HIV-1 to hide and not been detected by effector cells like CD8 T cells. In secondary lymphatic tissue, the B cell follicle is surrounded by an extrafollicular zone where CD8+ T cells can accumulate during HIV-infection (152, 153). To enter the B cell follicle, cells need to express the follicular homing receptor CXCR5, which makes the follicle an immune privilege site, including T_{FH} cells or B cells (153).

The probable biological purpose of this immune privilege site is to allow B cells and T_{FH} cells to interact in the germinal center in the absence of antigen clearance for optimal humoral immune responses (154). Although recent studies demonstrated that specific CD8 T cells have the ability to enter the B cell follicle, the majority of CD8 T cells fail to enter due to the lack of CXCR5 expression (155). In addition, it has been shown that CD8 CXCR5+ T cells in humans often are not HIV-specific, contain less cytolytic molecules, and are less polyfunctional suggesting an impairment of the effector function to clear an infection in this compartment (152, 156).

Due to the lack of clearance within the B cell follicle, HIV-1 persists indefinitely as an integrated part of the genome in a small population of latently infected CD4 T cells in these reservoirs. Although an effective therapy with antiretroviral drugs increases the life expectancy of HIV-infected individuals and can suppress HIV viremia to undetectable blood-levels, HIV can still not be eradicated. Therefore, new strategies have tried to overcome this issue by forcing latently infected cells to produce HIV proteins in the presence of antiretroviral therapies. These “shock and kill” strategies aim to use small molecule latency reversing agents (LRA) to activate virus transcription and viral protein production. In theory, latently infected cells are forced to produce viral proteins that become visible for the immune system (157). So far, clinical trials have demonstrated that LRAs were able to activate and increase HIV transcription from

latently infected cells effectively. Still, none of these studies have demonstrated a reduction in the pool of latently infected cells (158-160).

1.4 Aim of the thesis

The overall goal of this PhD thesis was to analyze cytolytic T cells in HIV-1 infection. Therefore, CD8 T cells under the impact of antiretroviral medication and cytolytic CD4 T cells with the ability to enter the B cell follicle were investigated.

Although HIV-specific CD8 T cells effectively control HIV-infection, they fail to clear infection even in the presence of ART and cure strategies such as “shock-and-kill”. Little is known how ART contributes to HIV-specific CD8 T cell function and the ability to clear HIV infection. Therefore, in the first part of this thesis, we decided to investigate the impact of different ART regimens *ex-vivo* and *in-vitro* on the function of CD8 T cells to proliferate, migrate, express effector cytokines and to kill virally infected cells. It is important to understand whether the choice of ART can have a significant impact on CD8 T cell effector functions, which may lead to implications for eradication strategies.

The second part of this thesis was focused on the possibility of cytolytic CD4 T cells to enter the B cell follicle and kill latently infected T_{FH} cells. It has been demonstrated that the lymphoid B cell follicle is an important viral reservoir harboring high levels of HIV infection even when HIV is undetectable in the periphery. It is believed that the B cell follicle is an immune privileged site blocking HIV-specific CTLs to enter, while T follicular helper cells can recirculate in and out of the follicle. Therefore, we decided to analyze CD4 T cells in the blood and secondary lymphoid tissue to identify and characterize cells with follicular homing properties and the ability to degranulate and secrete cytolytic molecules upon stimulation. Investigation of this cellular subset may provide a novel strategy for HIV eradication attempts.

2 Material and Methods

Not listed materials, antibodies, software or devices were mentioned in detail in the method section.

2.1 Devices

Table 1: Devices

Product	Manufacturer
AxioObserver.Z1	Carl Zeiss, Germany
BD FACSCelesta	BD Bioscience, Germany
BD FACSAria III	BD Bioscience, Germany
Carbone dioxide incubator	BINDER, Germany
Cellometer Auto T4	Nexcelom Bioscience, USA
Centrifuge (Eppendorf 5804R)	Eppendorf, Germany
EVE cell counter	NanoEntek, Korea
Liquid nitrogen tank	Techlab, Germany
NanoQuant Infinite M2000	TECAN, Switzerland
Seahorse XFe 96 Analyzer	Agilent, USA
Test tube vortexer	NeoLab Migge, Germany
Water bath GFL	Oehmen, Germany

2.2 Materials

Table 2: Materials

Product	Manufacturer
15/50ml falcon tubes	TPP, Switzerland
96-well microplates	Starlab, Germany
24-well microplates	Starlab, Germany
6-well microplates	Starlab, Germany
384 µ-well plate	Corning, USA
Cell culture flasks T75	Greiner Bio One, Germany
Cell strainer (40µm)	TPP, Switzerland
Cryotubes	Oehmen, Germany

Cryo-freezing container	Sigma-Aldrich, Germany
DynaMag2	Thermo Fischer Scientific, Germany
EVE counting slides	NanoEntek, Korea
FACS tubes	BD Bioscience, Germany
MACS LS columns	Miltenyi, Germany
MACS separator magnet	Miltenyi, Germany
Microtubes 1,5/2ml	Eppendorf, Germany
Pipettes (5, 10, 25ml)	Greiner Bio One, Germany
Pipette filter tips	Starlab, Germany
pH meter	Oehmen, Germany
Seahorse cartridge	Agilent, USA
Seahorse microplate	Agilent, USA

2.3 Chemicals

Table 3: Chemicals

Product	Manufacturer
anti-CD3/CD28 beads	Gibco, Life Technologies, UK
Antimycin A	Agilent, USA
Biocoll (ficoll)	BioChrome, Germany
BSA	Sigma-Aldrich, Germany
Carboxyfluorescein succinimidyl ester	Biolegend, USA
CD28/CD49d	BD Bioscience, Germany
CytoFix/CytoPerm Solution	BD Bioscience, Germany
Darunavir ethanolate	Selleckchem, USA
DMSO	Sigma-Aldrich, Germany
Dolutegravir	Selleckchem, USA
EDTA	Sigma-Aldrich, Germany
Elvitegravir	Selleckchem, USA
Emtricitabine	Selleckchem, USA
FBS	BioChrome, Germany
FCCP	Agilent, USA
Glucose	Agilent, USA

Golgi Stop and Golgi Plug	BD Bioscience, Germany
Hematopoietic progenitor growth media	Lonza, Switzerland
HEPES	Sigma-Aldrich, Germany
Human T cell activator	Thermo Fischer Scientific, Germany
ICAM-1	R&D Systems, USA
IL-2	eBioscience, USA
Lamivudine	Selleckchem, USA
L-Glutamine	Sigma-Aldrich, Germany
Oligomycin	Agilent, USA
Nevirapine	Selleckchem, USA
10x Non-Essential Amino Acids Solution	Thermo Fischer Scientific, Germany
PBS	Life Technologies, UK
Penicilline	Sigma-Aldrich, Germany
10x Perm/Wash Buffer	BD Bioscience, Germany
Phytohaemagglutinin	Remel, USA
Poly-D-Lysine	Sigma-Aldrich, Germany
Raltegravir	Selleckchem, USA
Rilpivirine	Selleckchem, USA
RPMI 1640 medium	Life Technologies, UK
Rotenone	Agilent, USA
Ritonavir	Selleckchem, USA
SDF-1α	Peprotech, USA
Serum Replacement 3	Sigma-Aldrich, Germany
Sodium pyruvate	Sigma-Aldrich, Germany
Staphylococcus entrotoxin B	Sigma-Aldrich, Germany
Streptomycin	Sigma-Aldrich, Germany
Tenofovir-disoproxil	Selleckchem, USA
Trypan Blue 0.4%	NanoEn Tek, USA
Zidovudine	Selleckchem, USA

2.4 Commercial kits

Table 4: Commercial kits

Product	Manufacturer
CD4 T Cell Isolation Kit	Miltenyi, Germany
CD8 T Cell Isolation Kit	Miltenyi, Germany
CD8 T cell Multisort Isolation Kit	Miltenyi, Germany
MACSxpress Whole Blood CD8 T Cell Isolation Kit	Miltenyi, Germany
MACSxpress® Erythrocyte Depletion Kit	Miltenyi, Germany

2.5 Methods

For the purpose of this work, HIV-1 will from now on be referred to as HIV.

2.5.1 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

EDTA-supplemented blood samples were obtained from healthy donors, HIV positive treated and treatment-naïve individuals and blood mononuclear cells were isolated by standard density-gradient protocol and cryopreserved as previously described (161). Briefly, blood was mixed with RPMI-1640 supplemented with 10 % heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicilline, 100 µg/ml streptomycine as well as 10 mM HEPES (R10 media), was pipetted on a ficoll layer and subsequently centrifuged at 900 xg for 35 min. Afterwards, the layer containing PBMCs was transferred into a new falcon tubed, washed twice with 30 ml of R10 media and centrifuged at 600 xg for 10 min. Cells were then cryopreserved in 10 % dimethylsulfoxid (DMSO) and 90 % FCS or directly used for further experiments.

2.5.2 Isolation of mononuclear cells from lymph nodes and tonsils

Lymph nodes and tonsils were obtained from healthy donors as well as lymph nodes from HIV-infected individuals and lymphatic mononuclear cells were isolated. Briefly, lymphatic tissue was placed in a cell strainer and afterwards positioned in a petri dish with R10 media inside. The lymphatic tissue was grounded in the cell strainer; in that way tissue remained in the strainer and cells were washed in the R10 media. Afterwards, the R10 media with cells was collected into a new falcon tube and washed

twice with 30 ml of R10 media and centrifuged at 600 xg for 10 min. Cells were then cryopreserved in 10 % DMSO and 90 % FCS or directly used for further experiments.

2.5.3 Cell subset separation and stimulation conditions

Cryopreserved PBMCs were thawed in R10 media and allowed to rest overnight at 37 °C, 5 % CO_2. CD8 T cells were subsequently enriched by magnetic separation using the CD8 T Cell Isolation Kit, achieving more than 90 % purity. Cells were then stimulated using anti-CD3/CD28 beads at a cell-to-bead ratio of 1:1 in the presence of IL-2 (50 U/ml) for three days. Cells were cultured in R10 media in a humidified atmosphere (5 % CO_2) at 37 °C. To assess the impact of antiretroviral medications, CD8 T cells were incubated in the presence of different ART regimens – emtricitabine (FTC), zidovudine (AZT), tenofovir-disoproxil (TDF), lamivudine (3TC), rilpivirine (RLP), raltegravir (RAL), dolutegravir (DTG), elvitegravir (EVG), darunavir ethanolate (DRV), ritonavir (RTV) at 5-fold or bioactive plasma concentration. The concentration of each antiretroviral medication is described in (**Table 7**).

2.5.4 Assessment of ex-vivo polyfunctionality by flow cytometry

Cryopreserved PBMC from HIV+ treated individuals were thawed and rested overnight as described above. Cells were then stimulated with 5 µg/ml of the staphylococcus entrotoxin B (SEB) and incubated in the presence of the co-stimulatory molecule CD28/CD49d together with anti-CD107a-PE/Dazzle 594 (clone H4A3; Biolegend) at 37 °C, 5 % CO_2 for six hours. An unstimulated condition served as control. After one hour, Golgi Stop and Golgi Plug (BD Bioscience) were added and cells were incubated for additional five hours. Cells were then washed with phosphate buffered saline (PBS) and stained with viability dye Zombie Aqua (Biolegend). After washing with staining buffer (PBS supplemented with 2 % FCS), cells were stained extracellularly for 20 min with the fluorescently conjugated antibody anti-CD8-PE-Cy7 (clone RPA-T8; Biolegend). After washing with staining buffer, CytoFix/CytoPerm solution was added and cells were incubated for 20 min at 4 °C. Cells were then washed with 1x Perm/Wash Buffer and stained intracellularly with fluorescently conjugated antibodies: anti-CD3-APC-Cy7 (clone UCHT1; Biolegend), anti-CD4-AF700 (clone RPA-T4; Biolegend), anti-IFNγ-APC (clone B28; Biolegend), anti-TNFα-BV421 (clone Mab11; Biolegend), anti-MIP1β-PE (clone D21-1351; BD Bioscience) for 30 min at 4 °C. Data were collected at FACS Celesta (BD) and analyzed with the FlowJo Software version 10.0.7 (TreeStar).

2.5.5 Assessment of *ex-vivo* proliferation by flow cytometry

Cryopreserved PBMC from HIV-infected, ART-treated individuals were thawed and rested overnight as described above. Afterwards, approximately 3 million PBMCs from each individual were washed with PBS and stained with 1.25 µM of the cell division tracker carboxyfluorescein succinimidyl ester (CFSE) in 1 ml PBS at 37 °C, 5 % CO_2 for 20 min. Subsequently, the staining was quenched by adding 5 times the original staining volume of R10 media. Cells from each individual were centrifuged, resuspended in R10 media and transferred in a 24-well polystyrene plate at concentration of 1 million/ml. Cells were stimulated with 5 µg/ml SEB or left unstimulated as a control at 37 °C, 5 % CO_2 for five days. On the 5th day, cells were washed with PBS and stained with the viability dye Zombie Aqua (Biolegend) for 30 min. After washing with staining buffer (PBS supplemented with 2 % FCS), cells were stained extracellularly for 20 min with fluorescently conjugated antibodies: anti-CD3-APC-Cy7 (clone UCHT1; Biolegend), anti-CD4-BV421 (clone RPA-T4; Biolegend), anti-CD8-AF647 (clone C8/144B; Biolegend). Data were collected at FACS Celesta (BD) and analyzed with the proliferation tool of FlowJo Software version 10.0.7 (TreeStar).

2.5.6 T cell migration assay

CD8 T cells were isolated from 7 ml EDTA-supplemented HIV negative blood via negative magnetic separation using the MACSxpress® Whole Blood CD8 T Cell Isolation Kit. Residual erythrocytes were removed afterwards with an additional magnetic separation step using the MACSxpress® Erythrocyte Depletion Kit. Next, isolated CD8 T cells were washed with 10 ml sterile PBS, resuspended in sterile hematopoietic progenitor growth medium (HPGM) supplemented with 0.3x sterile Serum Replacement 3, 1x sterile Non-Essential Amino Acids Solution as well as 1 mM sodium pyruvate and counted using a Cellometer Auto T4. Cells were transferred to an ICAM-1-coated (5 µg/ml per well) hydrophobic, optical imaging, tissue-culture treated 384 µ-well plate at a density of 2.500 cells per well. To assess the impact of antiretroviral medications, CD8 T cells were incubated in the presence of DMSO or different ART regimens- emtricitabine (FTC), dolutegravir (DTG), darunavir ethanolate (DRV) and rilpivirine (RLP) at previously described bio-active plasma concentrations at 37 °C, 5 % CO_2 for one day. After 24 h, cells were stimulated with PBS (control) or 500 ng/ml SDF-1α and imaged on an AxioObserver.Z1 with a motorized stage, 20x magnification and a rate of one frame/15 s for 3 h at 37 °C. Generated Multi-TIFF files

were converted to *.mov files. With these files, automated segmentation and tracking were performed using the Automated Cellular Analysis System (ACAS, MetaVí Labs). The parameter presented in this manuscript is the percentage of moving cells upon stimulation.

2.5.7 Assessment of T cell polyfunctionality

Cryopreserved PBMC isolated from healthy donors were thawed and rested overnight as described above. Cells were transferred in a 24-well polystyrene plate at a concentration of 1 million/ml and stimulated with 200 ng/ml SEB in the presence of different ART regiments at previously described bio-active plasma concentrations at 37 °C, 5 % CO_2 for three days. On day three, cells were re-stimulated with 5 µg/ml SEB, and incubated in the presence of co-stimulatory molecule CD28/CD49d together with anti-CD107a-PE-Cy5 (clone H4A3; BD Bioscience) for six hours. After one hour, Golgi Stop and Golgi Plug (BD Bioscience) were added and cells were incubated for additional five hours. Cells were then washed with PBS and stained with viability dye Zombie Aqua (Biolegend). After washing with staining buffer (PBS supplemented with 2 % FCS), CytoFix/CytoPerm Solution was added and cells were incubated for 20 min at 4 °C. Cells were then washed with 1x Perm/Wash Buffer and stained intracellularly with fluorescently conjugated antibodies: anti-CD3-PacificBlue (clone UCHT1; Biolegend), anti-CD4-BV421 (clone RPA-P4; Biolegend), anti-CD8-APC-Cy7 (clone RPA-T8; Biolegend),anti-MIP1β-PE (clone D21-1351; BD Bioscience), anti-TNFα-AF700 (clone Mab11; Biolegend), anti-IFNγ-PECy7 (cloneB27; Biolegend), anti-IL-2-FITC (clone MQ1-17H12; Biolegend) for 30 min at 4 °C. Data were collected and analyzed as above.

2.5.8 Viral inhibition assay

Cryopreserved PBMC isolated from HIV infected treatment-naïve individuals were thawed and rested overnight as described above. CD8 T cells were subsequently isolated by positive magnetic separation using CD8 T cell Multisort Isolation Kit and CD4 T cells were isolated by negative magnetic separation using CD4 T Cell Isolation Kit. After isolation, CD4 T cells and CD8 T cells were resuspended in R10 media supplemented with IL-2 (50 U/ml) and transferred to a 48-well polystyrene plate at concentration of 5 x 10^5/ml per well. Additionally, CD8 T cells were incubated in the presence of DMSO or different ART regimens- emtricitabine (FTC), dolutegravir (DTG) and rilpivirine (RLP) at 5-fold plasma concentration at 37 °C, 5 % CO_2 for three days.

On day three, CD4 T cells were spinoculated at 1200 ×g for 1 h with HIV nevirapine-resistant containing supernatant (NIH AIDS Reagents, Bethesda, USA) and subsequently incubated at 37 °C, 5 % CO_2 for 2 h. Afterwards, the virus was washed off the cells. At the same time, CD8 T cells were washed six times with sterile PBS to ensure the ART medication was washed off and afterwards cells were co-cultured in R10 media supplemented with IL-2 (50 U/ml) and nevirapine in a ratio of 1:2 (effector: target ratio) with the nevirapine-resistant virus-infected autologous CD4 T cells. At day 3 and 7 post-infection, an HIV-1 Gag p24 Quantikine ELISA was performed according to the manufacturer`s instructions. The ELISA plate was measured with a NanoQuant Infinite M2000 plate reader.

2.5.9 Assessment of T cell cytolytic phenotype

Cryopreserved PBMCs isolated from healthy donors were thawed and rested overnight as described above. Cells were transferred to a 24-well polystyrene plate at a concentration of 1 million/ml per well and either stimulated with 200 ng/ml SEB or left unstimulated in the presence of different ART regiments at a 5-fold plasma concentrations at 37 °C, 5 % CO_2 for three days. On day three, cells were re-stimulated with 5 µg/ml SEB, and incubated in the presence of co-stimulatory molecule CD28/CD49d together with anti-CD107a-PE/Dazzle 594 (clone H4A3; Biolegend) for six hours. After one hour, Golgi Stop and Golgi Plug were added and cells were incubated for additional five hours. Cells were then washed with PBS and stained with viability dye Zombie Aqua (Biolegend). After washing with staining buffer (PBS supplemented with 2 % FCS), cells were stained with the fluorescently conjugated antibody anti-CD8-AF647 (clone C8/144B; Biolegend) for 20 min at 4 °C and subsequently washed again with staining buffer. Afterwards, CytoFix/CytoPerm Solution was added and cells were incubated for 20 min at 4 °C. Cells were then washed with 1x Perm/Wash Buffer and stained intracellularly with fluorescently conjugated antibodies: anti-CD3-APC-Cy7 (clone UCHT1; Biolegend), anti-CD4-AF700 (clone RPA-P4; Biolegend), anti-Granzyme-B-BV421 (clone GB11; BD Bioscience), anti-Perforin-PeCy7 (clone B-D48; Biolegend) for 30 min at 4 °C. Data were collected at FACS Celesta (BD) and analyzed with FlowJo (Version 10.1) as described above.

2.5.10 Assessment of the phenotype of cytolytic CD4 T cells with follicular homing properties

Cryopreserved PBMCs or lymphatic mononuclear cells isolated from healthy donors or HIV-infected individuals were thawed and rested overnight as described above. Cells were transferred to round-bottom polystyrene tubes at a concentration of 1 million/ml per well and either stimulated with 200 ng/ml SEB or left unstimulated and incubated in the presence of the co-stimulatory molecule CD28/CD49d together with anti-CD107a-PE/Dazzle 594 (clone H4A3; Biolegend) for six hours 37 °C, 5 % CO_2. After one hour, Golgi Stop and Golgi Plug were added and cells were incubated for additional five hours. Cells were then washed with PBS and stained with viability dye Zombie Aqua (Biolegend). After washing with staining buffer (PBS supplemented with 2 % FCS), cells were stained with the fluorescently conjugated antibodies: anti-PD1-BV786 (clone EH12.2h7; Biolegend), anti-CTLA4-BV605 (clone BNI3; Biolegend), anti-CXCR5-AF488 (clone J252D4; Biolegend) for 20 min at 4 °C. Afterwards, cells were washed again with staining buffer and CytoFix/CytoPerm solution was added to the cells which were incubated for 20 min at 4 °C. Cells were then washed with 1x Perm/Wash Buffer and stained intracellularly with fluorescently conjugated antibodies: anti-CD3-APC-Cy7 (clone UCHT1; Biolegend), anti-CD4-AF700 (clone RPA-P4; Biolegend), anti-Granzyme-B-BV421 (clone GB11; BD Bioscience), anti-Perforin-PeCy7 (clone B-D48; Biolegend), anti-IFNγ-PE (clone B27; Biolegend) for 30 min at 4 °C. Data were collected at FACS Celesta (BD) and analyzed with FlowJo (Version 10.1) as described above.

2.5.11 Metabolic assay

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were determined using a Seahorse XFe Extracellular Flux Analyzer according to the manufacturer´s protocol. Briefly, cells were plated on poly-D-lysine coated 8-well polystyrene Seahorse plate at a density of 200.000 cells per well. Cells were equilibrated in non-buffered DMEM medium supplemented with 2 mM glutamine, 2 mM sodium pyruvate and 10 mM glucose for 45 min at 37 °C with 0 % CO_2 atmosphere prior to the experiment. Metabolic assays were performed using the following concentrations of subsequently injected compounds: oligomycin 1 µM, mitochondrial uncoupler p-trifluormethoxy carbonyl cyanice phenol hydrazine (FCCP) 1 µM, Antimycine A and rotenone (both 0.5 µM). At each time interval, the OCR and ECAR

values were measured as indicators mitochondrial respiration and glycolysis, respectively.

2.5.12 Assessment of biological, antiviral activity in vitro

Cryopreserved PBMCs isolated from healthy HIV-negative donors were thawed, rested overnight and activated with 1 µg/ml of phytohaemagglutinin (PHA) for three days. They were then seeded in a 6-well polystyrene plate at a density of 1.5 x 10^6 cells/ml and infected with 67 ng/ml of HIV-1 US-1 virus (NIH AIDS Reagents, Bethesda, USA). A 5-fold plasma concentration of different ART regimens (DTG, RAL, EVG or DMSO as control) was added into the suspension and cells were incubated at 37 °C, 5 % CO_2 for another three and four days. Cells were harvested, washed with PBS and stained with viability dye Zombie Aqua (Biolegend) for 30 min at RT. Cells were subsequently washed with staining buffer (PBS supplemented with 2 % FCS) and stained with fluorescently conjugated antibody anti-CD8-AF647 (clone RPA-T8; Biolegend) for 20 min at 4 °C. Performing the intercellular staining, cells were washed again with staining buffer and incubated with CytoFix/CytoPerm solution for another 20 min at 4 °C. After washing with 1x Perm/Wash Buffer, cells were intracellularly stained with fluorescently conjugated antibodies: anti-CD3-APC-Cy7 (clone UCHT1; Biolegend), anti-CD4-BV421 (clone RPA-P4; Biolegend) and anti-p24-PE (clone KC57; Beckman coulter) for 30 min at 20°C. Data were collected at FACS Celesta (BD) and analyzed with FlowJo (Version 10.1) as described above.

2.5.13 Assessment of T cell viability

The viability of T cells was repeatedly assessed by using trypan blue staining. Briefly, 10 µl of cell suspension was mixed with 10 µl of 0.4 % trypan blue and loaded onto a cell counting slide. Afterwards, the number of live cells was determined using an automatic cell counter EVE. To determine whether living cells undergo early apoptosis cells were stained repeatedly with Annexin V. Cells were washed with staining buffer (PBS supplemented with 2% FCS) and stained extracellularly for 20 min with fluorescently conjugated antibodies: anti-CD3-APC-Cy7 (clone UCHT1; Biolegend), anti-CD4-BV421 (clone RPA-T4; Biolegend). Afterwards, cells were washed with Annexin V Binding Buffer (Biolegend) and stained with anti-AnnexinV-FITC (Biolegend) antibody for 15 min. Subsequently, 400 µl of Annexin V Binding Buffer were added to the cells. Data were collected at FACS Celesta (BD) and analyzed with FlowJo Software version 10.0.7 (TreeStar).

2.5.14 Statistical Analysis

Prism (GraphPad Software) was used for statistical analysis. Data are presented as mean ± SD for technical replicates or mean ± SEM for biological replicates. Differences between treatment categories were estimated with ANOVA or student t test. Statistical significance is indicated in all figures by the following annotations: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3 Results

3.1 Impact of antiretroviral therapy regiments on CD8 T cell activity and function

Although HIV-specific CD8 T cells effectively control HIV-infection, they fail to clear infection even in the presence of ART and cure strategies such as “shock-and-kill”. Little is known how ART contributes to HIV-specific CD8 T cell function and the ability to clear HIV infection. Here, the impact of different ART regimens on the function of CD8 T cells were assessed to proliferate, migrate, express effector cytokines and kill virally infected cells.

3.1.1 Reduced functionality in individuals treated with INSTI-based regimens *ex-vivo*

To understand the impact of ART on CD8+ T cell function, the *ex vivo* CD8 T cell functionality in individuals that had been on antiretroviral therapy for more than six months and had fully suppressed viremia (HIV RNA detection <50 copies/ml) were assessed. Therefore, PBMCs from 36 ART-treated individuals were used with fully suppressed viremia that either received an INSTI-, NNRTI- or PI-containing ART regimen (**Table 5**). All participants in this study had two NRTI as a backbone in combination with a third drug from INSTI, NNRTI or PI class. Most of INSTI users received DT G in their ART regimen. All of NNRTI users received RLP and the majority of PI users received RTV in combination with DRV. The cells were stimulated with SEB for six hours and afterwards analyzed the functionality of CD8 T cells using a polyfunctionality panel consisting of four functional markers.

Table 5: List of participants and their regiments in the *ex-vivo* polyfunctional study

(A) Characteristics of individuals used in the *ex vivo* polyfunctional assay. Plus-minus values are means ± SD. (B) Individual ART regimens. All participants in this study had two NRTI as a backbone combined with a third drug from INSTI, NNRTI or PI class listen in first column.

A

Individuals Ex-Vivo Polyfunctional

Demographics	Overall	INSTI	NNRTI	PI	P-value
Age					0.0554
Mean ± std	46.1 ± 13.9	44.9 ± 13.1	41.2 ± 12.5	54.1 ± 13.7	
Gender					
Male	34	11	13	10	
Female	2	2	0	0	
CD4 count*					0.219
Median	615.2 ± 209.0	708.5 ± 296.3	550.9 ± 106.7	577.5 ± 132.1	
CD8 count*					0.7676
Median	691.3 ± 209.1	671.5 ± 249.1	727.2 ± 179.9	670.5 ± 203.2	
Days on treatment					0.0036
Mean ± std	915.1 ± 690.9	588.7 ± 280.6	681.7 ± 393.1	1642.9 ± 851.0	

*at the time the sample was taken

B

INSTI		n
DTG	ABC+3TC	10
EVG	FTC+TAF	3
	total:	13
NNRTI		
RLP	FTC+TAF	3
RLP	FTC+TDF	10
	total:	13
PI		
RTV+DRV	FTC+TDF	9
LPV	FTC+TDF	1
	total:	10

For our *ex-vivo* polyfunctional as well as proliferation study, we decided to use the superantigen SEB to evoke a strong response and detect even slight differences between the different ART regimens. It has been shown that HIV-infected individuals on long term treatment have a reduction in the magnitude and breath of their HIV-specific CD8 T cell response. Viremia is one of the main drivers for the magnitude of HIV-specific response. Therefore, it was shown that the magnitude of HIV-specific CD8 T cell response in untreated chronic infection is at least 2-fold greater than the

magnitude of HIV-specific CD8 T cells from a treated HIV infection (162). Given the fact that these individuals were on ART treatment for at least six months, we could detect in the past only a weak and insufficient HIV-specific CD8 T cell response in *ex-vivo* experiments using a Gag peptide pool as stimulus. For the detection of differences between the ART regimens, a stronger response is required, which we achieved by using a superantigen like SEB. CD8 T cells from all individuals responded well to SEB stimulation; however, we observed a striking perturbation in the composition of the responses depending on the ART regimen each individual received. The NNRTI and PI-based regimens showed a relatively similar polyfunctionality expression profile where the majority had the ability to simultaneously secrete 4 cytokines (CD107a, IFNγ, MIP1β and TNFα) followed by a 3 functional response with different cytokine combinations (**Figure 12**).

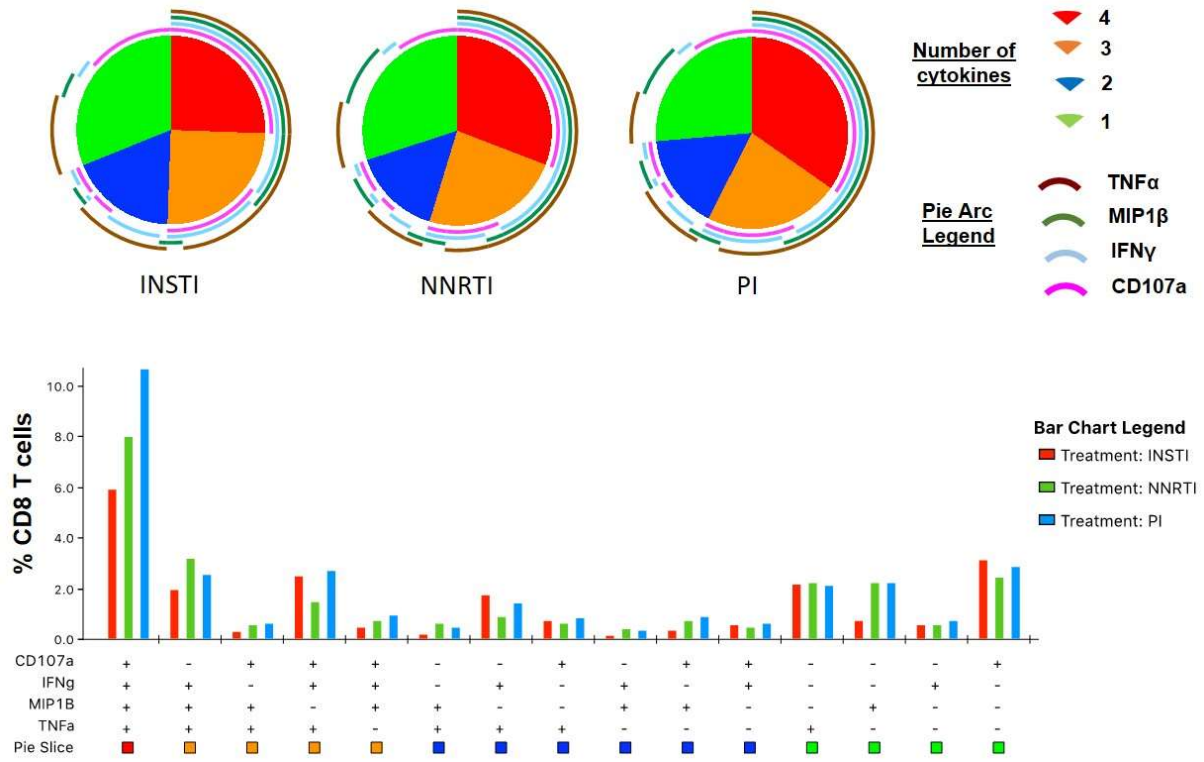

Figure 12: SPICE analysis showing a shift in the polyfunctionality profile of CD8 T cells.

A shift in the polyfunctionality profile of CD8 T cells from individuals receiving INSTI-containing regimen towards a less multifunctional response compared to individuals receiving NNRTI- and PI-based regimens was overserved. The pie charts represent the average frequencies of active cytokine-producing CD8 T cells producing every possible combination (pie-legend: green to red) of the four cytokines analyzed. The size of the pie segment correlates to the frequency of the particular population. The arcs around the circumference indicate the

particular cytokine produced by the proportion of cells that lie under the arc. The bar graph beneath the pie segment shows frequencies of combinations of cytokines.

This multifunctional response was significant decreased in individuals receiving an INSTI-based ART regimen upon SEB stimulation **(Figure 13)**. Despite a full-suppression of HIV viremia in all individuals, we observed that CD8 T cells from these individuals secreted a significantly lower amount of 4 cytokines simultaneously, compared to cells from individuals receiving NNRTI- ($p<0.05$) and PI-based regimens ($p<0.01$). Similarly, there was a significant reduction in the three-functional CD8 T cell response ($p<0.05$) in comparison to subjects that received a PI-based ART regimen. This was particularly striking as all assays were done *ex vivo* with no supplementation of ARVs or other potential confounding factors.

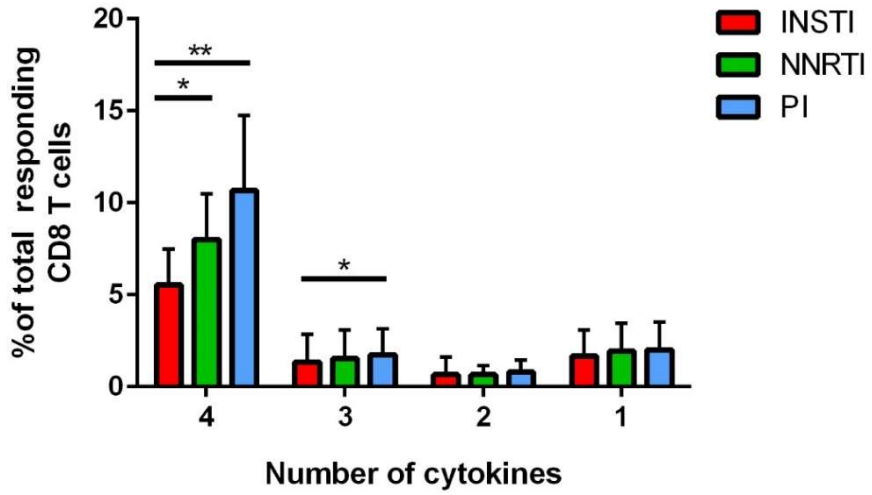

Figure 13: Polyfunctionality profile of CD8 T cells from individuals treated with different ART regimens.

CD8 T cells from of ART-treated HIV+ individuals receiving INSTI-containing regimen (red bar) secreted a significantly lower amount of 4 cytokines simultaneously, compared to CD8 T cells from individuals receiving NNRTI- (green bar) and PI-based (blue bar) regimens. Bar chart shows the mean value ± SD. Statistical significance was assessed by RM one-way ANOVA test with Holm-Sidak´s multiple comparison test. (*P<0.05; **P<0.01; ***P<0.001).

Thus, our data demonstrate significant differences in the multifunctional response of HIV-specific CD8 T cells *ex vivo*. Individuals who received an INSTI-based ART regimen showed a reduced ability to simultaneously secrete multiple cytokines compared to individuals on PI- or NNRTI based regimens.

3.1.2 Reduced proliferation capacity in individuals treated with INSTI-based regimens *ex-vivo*

Given the impaired responsiveness of CD8 T cells in individuals receiving INSTI-based ART regimens, we next assessed whether ART regimens also have an impact on CD8 T cell proliferation. We evaluated the *ex vivo* proliferative capacity of CD8 T cells from HIV-treated individuals that either received an INSTI-, NNRTI- or PI-containing ART regimen with two NRTI drugs as a backbone. All individuals had similar CD4 and CD8 counts and had fully suppressed viremia for more than six months (**Table 6**).

Table 6: List of participants and their regiments in the ex-vivo proliferation study.

(A) Characteristics of individuals used in the *ex vivo* proliferation assay. Plus-minus values are means ± SD. (B) Individual ART regimens. All participants in this study had two NRTI as a backbone in combination with a third drug from INSTI, NNRTI or PI class listen in the first column.

A

Individuals Ex-Vivo Proliferation

Demographics	Overall	INSTI	NNRTI	PI	P-value
Age					0.1469
Mean ± std	47.4 ± 12.5	47.1 ± 11.3	42.8 ± 12.4	53.0 ± 13.5	
Gender					
Male	42	18	13	11	
Female	4	3	0	1	
CD4 count*					0.1178
Median	564.1 ± 146.2	604.5 ± 145.8	512.5 ± 119.7	549.3 ± 163.2	
CD8 count*					0.6071
Median	645.7 ± 210.1	615.9 ± 187.6	663.0 ± 251.7	679.2 ± 210.2	
Days on treatment					0.007
Mean ± std	709.7 ± 696.5	392.9 ± 147. 9	617.6 ± 361.4	1363.8 ± 1065.0	

*at the time the sample was taken

B

INSTI		n
DTG	ABC+3TC	11
EVG	FTC+TAF	10
	total:	21
NNRTI		
RLP	FTC+TAF	1
RLP	FTC+TDF	12
	total:	13
PI		
RTV+DRV	FTC+TDF	9
RTV+ATV	FTC+TDF	2
RTV+ATV	FTC+TAF	1
	total:	12

We therefore used a CSFE proliferation assay where PBMCs were stained with a fixable-cell-permeant, fluorescein-based tracer to track cell division and cultured them in the presence of SEB for five days without any additional ART drugs. After five days, we observed significant differences in the proliferative capacity of CD8 T cells depending on the ART treatment choice. CD8 T cells from individuals who received

NNRTI- or PI-based regimens showed similar ability to proliferate and had no significant differences in their proliferation index (**Figure 14**). Strikingly, the ability of CD8 T cells to proliferate in individuals receiving an INSTI-based ART regimen (2.4±0.4) was significantly lower compared to the other two regimens, NNRTI (3.0±0.3; p<0.001) and PI (2.8±0.4; p<0.05).

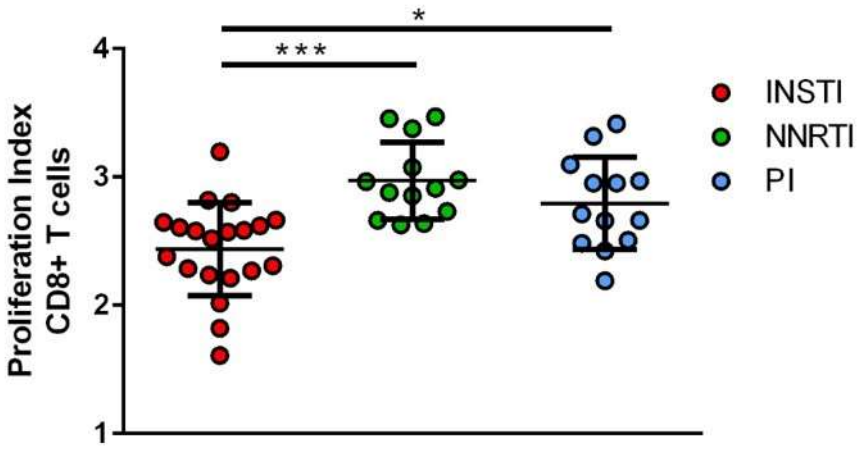

Figure 14: Assessment of the *ex vivo* proliferation capacity of CD8 T cells.

Proliferation capacity of CD8 T cells showed significantly decreased proliferation of CD8 T cells of ART-treated HIV+ individuals receiving INSTI-containing regimen (red circles) compared to NNRTI (green circles) and PI (blue circles) containing regimens. Chart shows the mean value ± SD. Statistical significance was assessed by RM one-way ANOVA test with Holm-Sidak´s multiple comparison test. (*P<0.05; **P<0.01; ***P<0.001).

Taken together, these ex vivo data suggest that INSTI-based regimens have a negative impact on CD8 T cell proliferation compared to NNRTI- and PI-based regimens.

3.1.3 Negative impact of dolutegravir treatment on CD8 T cell mobility

After we saw reduced polyfunctionality and proliferation of CD8 T cells in individuals receiving and INSTI-based regimen, we next wanted to understand which CD8 T cells functions are impaired by INSTI treatment. Therefore, we performed time-lapse video microscopy on CD8 T cells to assess their motility *in vitro*. The usage of a live-cell imaging assay helped us investigate the effect or ARVs on the migratory capacity of CD8 T cells, isolated from healthy donors. Afterwards, cells were treated with each ARV individually for 24 h and placed on an ICAM-1 coated plate. The migration of individual CD8 T cells was tracked after CXCL12α (SDF-1α) stimulation with an auto-tracking software that provides trajectory plots and additional migration parameters

such as percentage of moving cells. Untreated CD8 T cells responded well to the chemotactic cytokine and showed an equally distributed migration pattern over time with an average accumulated distance of 64 µm (**Figure 15**) as well as a 55 % migration induction of the total seeded CD8 T cells (**Figure 16**). Treatment with an exemplary substance for other ART classes (NRTI: FTC, PI: DRV and NNRTI: RLP) did not significantly change the migration pattern or activity of CD8 T cells compared to the untreated control.

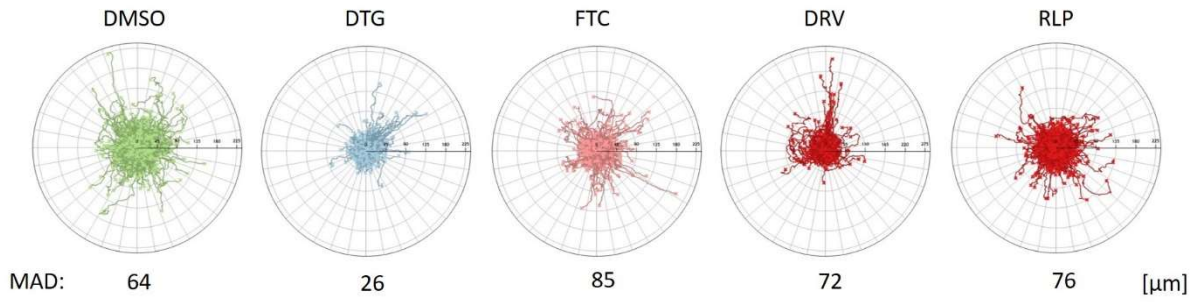

Figure 15: Assessment of the mobility of CD8 T cells treated with different ARTs individually.

Isolated CD8 T cells were cultured in the presence of DMSO or different ART regimens at bioactive plasma concentrations for one day and afterwards stimulated with SDF-1α. Migration of cells was recorded via video microscopy for a period of 3 h with one image taken every 15 s. The panel shows the exemplary trajectory plots (track origins set to 0.0) for the respective conditions generated using the ACAS auto-tracking tool. The scale bar indicates the distance between the ticks of the trajectory plots. The mean accumulated distance (MAD) is shown in µm.

However, we observed a significantly impaired migration pattern of CD8 T cells treated with the INSTI DTG. Interestingly, overall we observed that INSTI-treated cells showed less migration over time and had a significantly decreased percentage of moving cells (30.9±11.3), compared to DMSO (55.7±17.3; $p<0.05$) and NRTI FTC (54.1±14.3; $p<0.05$).

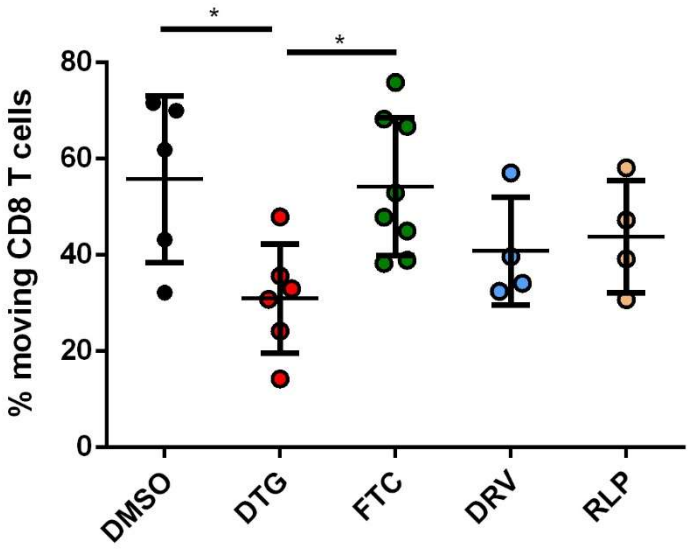

Figure 16: Assessment of the percentage of moving CD8 T cells treated with different ARTs individually.

Overall reduced percentage of moving CD8 T cells which were treated with DTG (red circles) compared to DMSO (black circles), FTC (green circles), DRV (blue circles) and RLP (yellow circles). Chart shows the mean value ± SD. Statistical significance was assessed by RM one-way ANOVA test with Holm-Sidak´s multiple comparison test. (*P<0.05)

Taken together, the data suggest that DTG interferes with the capability of CD8 T cells to migrate.

3.1.4 Dolutegravir and elvitegravir have a reductive effect on the cytokine expression profile of CD8 T cells

After we could determine differences in the polyfunctionality and proliferation of CD8 T cells in individuals receiving and INSTI-based regimen, we next wanted to understand the impact of individual antiretroviral medications and not the whole ART regimen. We therefore enriched CD8 T cells from HIV-negative donors and co-cultured them in the presence of individual ARVs at bioactive plasma concentrations for three days **(Table 7)**.

Table 7: List of tested ART drugs.

List of tested ART drugs with previously determined plasma concentration and concentration chosen in the assays.

Antiretroviral	Abbreviation	Plasma Concentration (ng/ml)		Chosen Concentration (ng/ml)	Source
		Median	Range		
Emtricitabine	FTC	717	21-1072	1000	Gish et al., 2002
Zidovudine	AZT	1150	710-1850	1500	Bergshoeff et al., 2004
Tenofovir DF	TDF	149	120-193	200	Avihingsanon et al., 2015
Tenofovir	TFV	NA*	NA*	200	
Lamivudine	3TC	2077	1264-2893	1500	Bruno et al., 2001
Rilpivirine	RLP	139	128-168	200	Dickinson et al., 2015
Raltegravir	RAL	448	37-5180	1000	Yilmaz et al., 2009
Dolutegravir	DTG	3908	3571-4245	4000	Elliot et al., 2016
Elvitegravir	EVG	1675	1557-1884	2000	Elliot et al., 2016
Darunavir ethanolate	DRV	3930	1800-12900	5000	Yilmaz et al., 2009
Ritonavir	RTV	746	646-1045	1000	Boffito et al., 2011

*variable plasma concentrations according to the prodrug

Afterwards, the multifunctional profile of the treated CD8 T cells was analyzed using a 4-marker functional panel after SEB stimulation. CD8 T cells stimulated in the presence of DMSO showed similar expression levels for CD107a, IFNγ, MIP1β and TNFα compared to a treatment with NRTI-, NNRTI- or PI-based drugs. There was no significant difference in the expression profile for these cytokines, except for ritonavir (RTV), which showed increased expression of CD107a compared to DMSO. However, stimulated CD8 T cells in the presence of INSTI-based regimens showed significant differences in the expression of IFNγ, MIP1β and TNFα. Cells stimulated in the presence of both INSTIs DTG and elvitegravir (EVG) but not raltegravir (RAL) demonstrated a significant decrease in the overall immune responses. In particular, CD8 T cells treated with DTG showed reduced functional properties with significantly lower expression of IFNγ ($p< 0.01$), MIP1β ($p< 0.001$) and TNFα ($p< 0.05$) compared to cells treated with DMSO **(Figure 15)**.

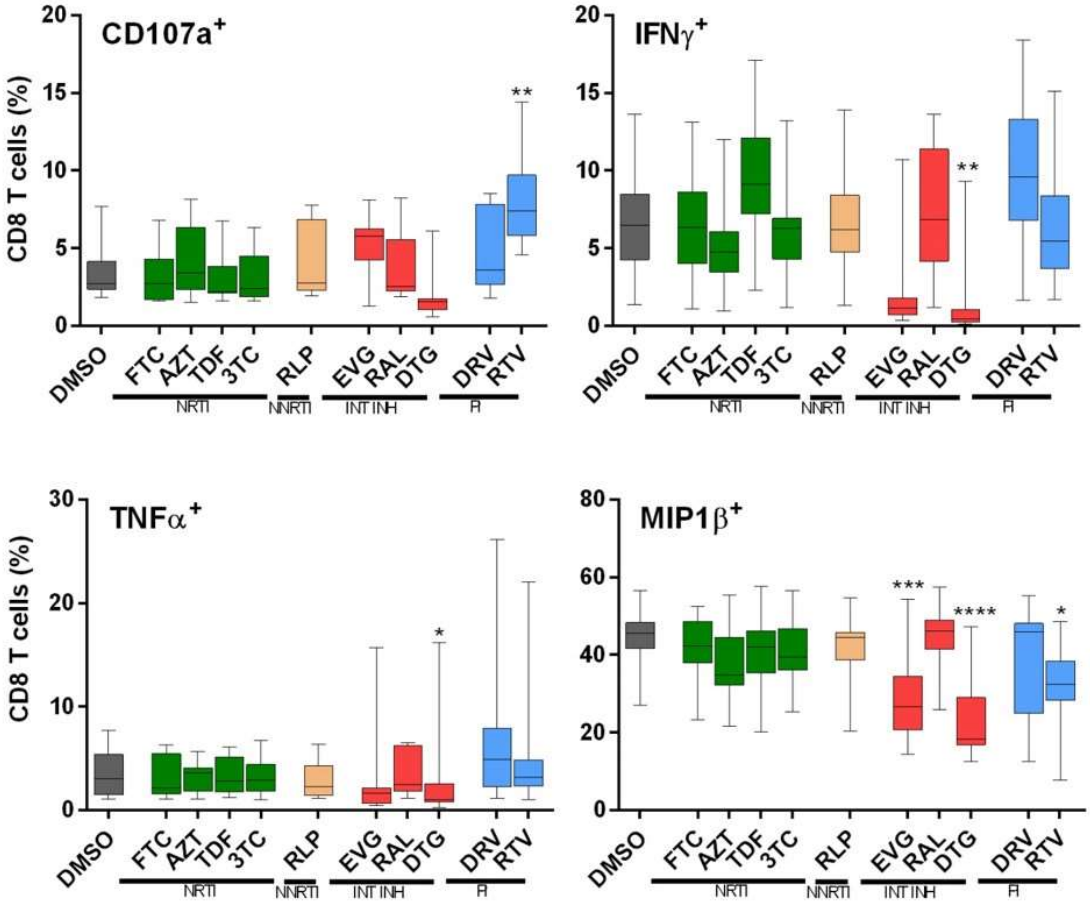

Figure 17: Assessment of CD8 T cell polyfunctionality after stimulation with SEB in the presence of different ARTs at bioactive plasma concentrations.

Cells were stained with fluorochrome-conjugated Abs specific for CD4, CD8, CD107a, IFNγ, MIP1β and TNFα and were analyzed by flow cytometry. Overall reduced functionality was observed in the presence of DTG and EVG for CD107a and significant for IFNγ, MIP1β and TNFα. Bar charts show the mean value ± SD. Statistical significance was assessed by RM one-way ANOVA test with Holm-Sidak´s multiple comparison test. (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).

In summary, only treatment with DTG and EVG resulted in a reductive effect on the cytokine expression of CD8 T cells.

3.1.5 Negative impact of dolutegravir on CD8 T cell cytolytic activity

Given the reduced function and mobility of CD8 T cells, we wanted to see if dolutegravir, as part of INSTI treatment, negatively affect the ability of CD8 T cells to kill virally infected cells. For this purpose, we analyzed how ARVs affect the inhibitory capacity of CD8 T cells in the presence or absence of the respective drugs using a viral inhibition assay. We therefore isolated CD8 T cells from HIV-infected treatment-

naïve individuals and treated them with the respective ARVs. Autologous treatment-naïve CD4 T cells from the same individuals were then infected with an NVP-resistant virus and co-cultured with CD8 T cells in the presence of NVP. It is important to note that the co-cultures were performed just in the presence of NVP and no other ARVs and only CD8 T cells had been previously exposed to other individual ARVs. Unexpectedly, CD8 T cells treated with DTG showed a similar killing ability seven days post-infection compared to the DMSO control and FTC or RLP treated cells. We found no reduction despite the previously shown impact of INSTI on T cell function (**Figure 18)**.

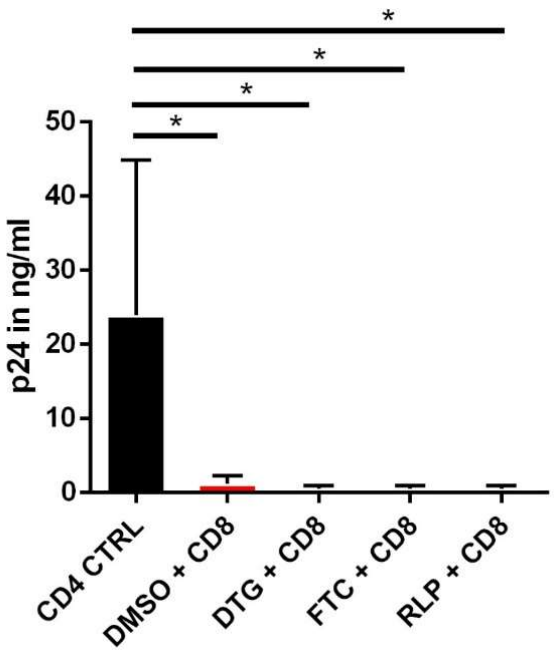

Figure 18: Assessment of the impact of individual ARTs on CD8 T cell cytolytic activity.

CD8 T cells from HIV infected treatment-naïve individuals (n=4) were isolated and incubated in the presence of DMSO or different ART regimens at 5-fold plasma concentrations for three days. Afterwards, CD8 T cells were co-cultured at a ratio of 1:2 (effector : target ratio) with previously nevirapine-resistant HIV virus infected autologous CD4 T cells. At day 3 and 7 post-infection, an HIV-1 Gag p24 Quantikine ELISA was performed to analyze the amount of p24 (in ng/ml) in the supernatant. No impact of any ART drug on CD8 T cells' ability to kill virally infected cells was overserved. Charts show the mean value ± SD. Statistical significance was assessed by RM one-way ANOVA test with Holm-Sidak´s multiple comparison test. (*P<0.05)

However, we hypothesized that DTG might affect the new synthesis of death molecules and therefore would have no impact on already existing, preformed molecules in the vesicles of the CD8 T cells. We therefore activated CD8 T cells from HIV-negative donors with SEB in the presence of different ARVs and subsequently measured their

expression of perforin and granzyme B. Strikingly, in resting and not activated CD8 T cells we could observe no significant effect of DTG on the expression of perforin and granzyme B after three days of treatment. These observations changed in previously activated CD8 T cells, we found the expression of both cytolytic molecules, perforin and granzyme B, to be significantly decreased in cells treated with DTG (51.6±25.1, 9.8±6.2, respectively) compared to FTC (99.3±0.9; p<0.01; 51.7±15.0; p<0.01 respectively) and RLP (44.6±22.1; p<0.05) treated cells **(Figure 19)**.

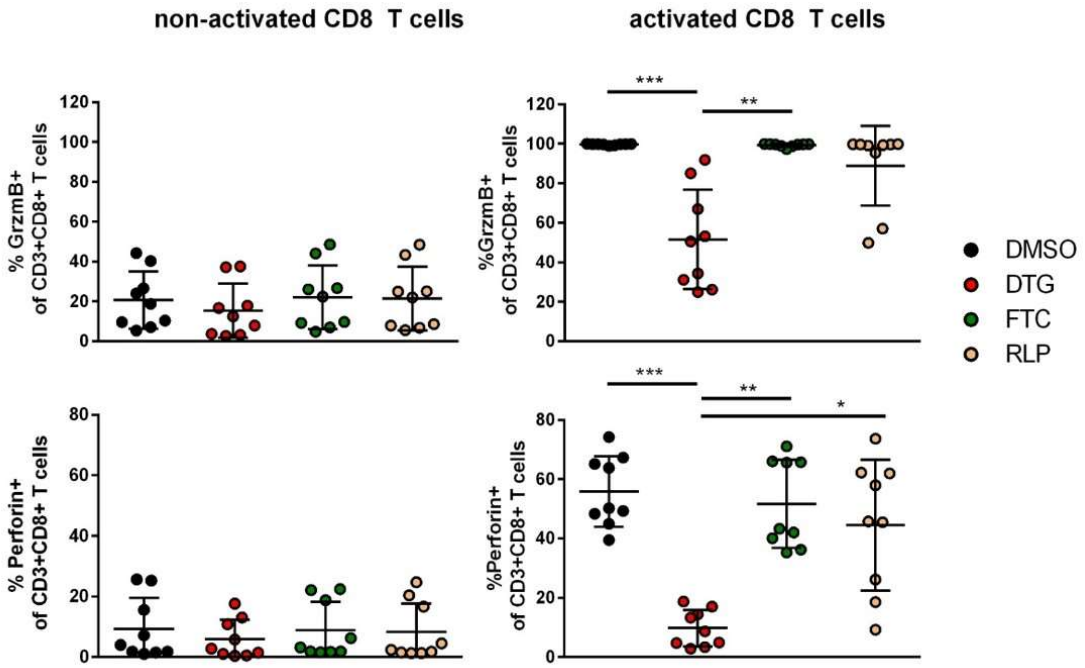

Figure 19: DTG effects the new synthesis of cytolytic molecules.

Isolated CD8 T cells from healthy individuals were either stimulated with 200 ng/ml SEB or left unstimulated in the presence of different ART regiments at 5-fold plasma concentrations for three days. Afterwards, cells were re-stimulated with 5 µg/ml SEB for six hours and analyzed via flow cytometry. A significant reduction in the expression of both cytolytic molecules perforin and granzyme B were observed in cells which were treated with DTG (red circles) compared to DMSO (black circles), FTC (green circles) and RLP (yellow circles) treated cells only in activated CD8 T cells. Charts show the mean value ± SD. Statistical significance was assessed by RM one-way ANOVA test with Holm-Sidak´s multiple comparison test. (*P<0.05; **P<0.01; ***P<0.001).

Overall, our data suggested that DTG has a negative impact on the cytolytic activity of activated CD8 T cells through reduced expression of the cytolytic molecules perforin and granzyme B.

3.1.6 Dolutegravir decreased mitochondrial respiration in CD8 T cells

To understand the underlying factors linked to the reduced function and activity of CD8 T cells, we next hypothesized that a change in the metabolic profile of the CD8 T cells could be a major reason. It has been recently reported that CD4 T cells, which were treated with INSTI, showed fundamental differences in their bioenergetics profile and T cell function (82). We therefore isolated CD8 T cells from HIV-negative donors and treated them with individual ARVs to understand the impact on CD8 T cell metabolic activity. We chose to determine differences in two major metabolic pathways – oxidative phosphorylation and glycolysis. Mitochondrial respiration was assessed by measuring the oxygen consumption rate (OCR) and aerobic glycolysis by measuring the acidification of the medium (extracellular acidification rate – ECAR) using an extracellular flux analyzer (Seahorse XFe). As expected, we could observe no significant impact on the metabolic activity in cells treated with the non-INSTI regiments FTC, RLP and DRV compared to cells treated with DMSO. However, DTG caused a significant decrease in basal respiration compared to DMSO, NRTI (FTC), NNRTI (RLP) and PI (DRV) treated CD8 T cells ($p<0.01$, $p<0.05$, $p<0.01$, $p<0.01$, respectively) and in maximal respiration ($p<0.05$, $p<0.05$; $p<0.01$, respectively). Interestingly, DTG had no impact on cellular glycolysis, suggesting that the inference of DTG specifically occur in the mitochondrial respiration pathway **(Figure 20)**.

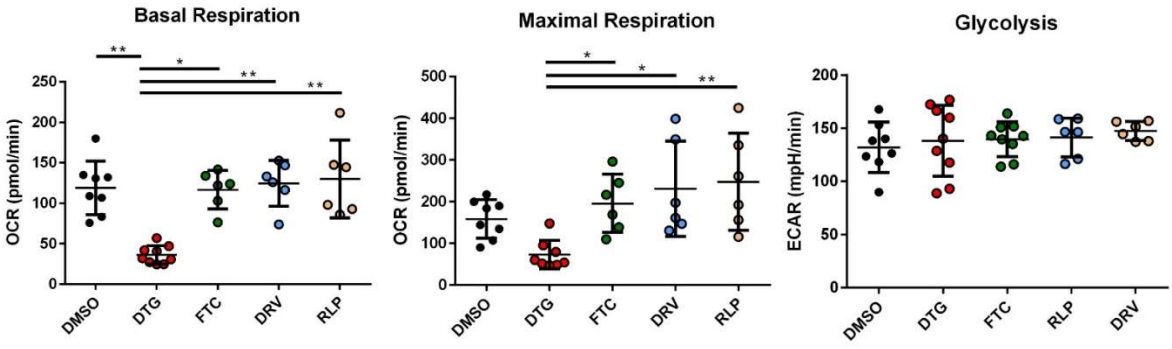

Figure 20: Assessment of the impact of individual ARTs on CD8 T cell metabolism.

Basal and maximal respiration of CD8 T cells, which were exposed to different ARTs at bioactive plasma concentrations for three days and analyzed using a Seahorse XFe Extracellular Flux Analyzer. Significant reduction in the oxygen consumption rate (OCR) was observed for CD8 T cells treated with DTG (red circles) compared to DMSO control (black circles), FTC (green circles), DRV (blue circles) and RLP (yellow circles). No differences in glycolysis were observed. Charts show the mean value ± SD. Statistical significance was assessed by RM one-way ANOVA test with Holm-Sidak´s multiple comparison test. (*P<0.05; **P<0.01).

Taken together, our data showed a negative influence of DTG on the metabolic activity of CD8 T cells.

3.1.7 Single regimens have no toxic impact on T cells

Next, we wanted to confirm that the observed impact of the individual ARVs on CD8 T cell function did not occur due to the fact of potential cellular cytotoxicity. We therefore isolated PBMCs from HIV-negative donors and treated them with individual ARVs at 5-fold plasma concentration for three days. Afterwards, we determined the cell viability by Trypan blue using a cell counter as well as by Annexin V using flow cytometry. There was no significant difference in the percentage of living cells regardless of the treatment **(Figure 21)**. Stained cells with Trypan blue and Annexin V showed a stable viability over a period of three days.

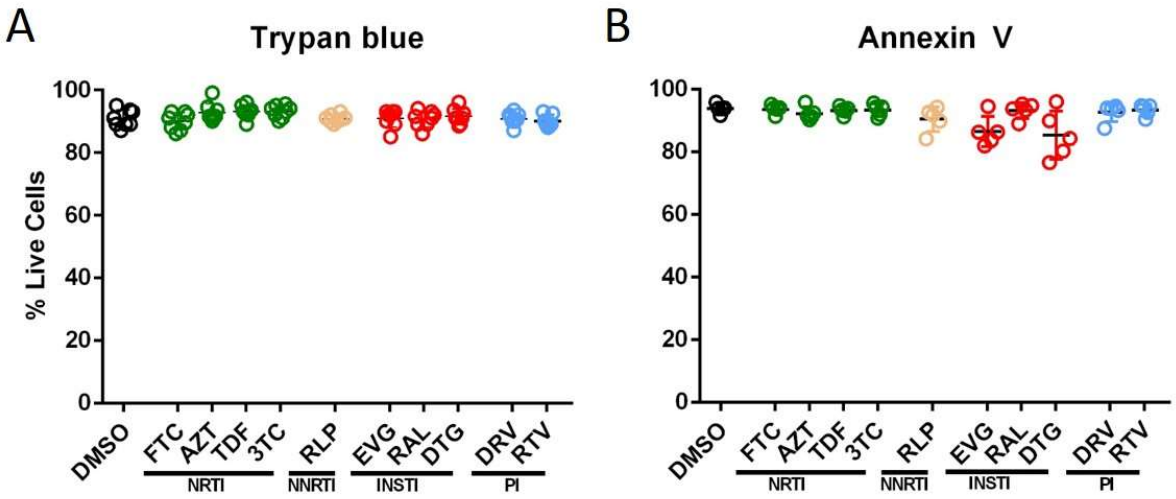

Figure 21: Assessment of cell viability in the presence of different ART regimens for three days.

(A) Viability assessed by Trypan blue and (B) by Annexin V. Plots show individual values with the mean ± SD.

Furthermore, we wanted to know if the used INSTI drugs in our in-vitro experiments were in their active form and had biological antiviral activity. Therefore, we isolated CD4 T cells from HIV-negative donors, infected them with an HIV-1 strain, treated them afterwards with either DMSO, DTG, EVG or RAL for three days and determined the percentage of p24+ CD4 T cells via flow cytometry. Untreated cells showed an increased amount of p24+ CD4 T cells, indicating high HIV infection of these cells

(Figure 22). CD4 T cells treated with all three drugs individually showed a decreased amount of p24+ T cells due to the fact of inhibiting HIV replication.

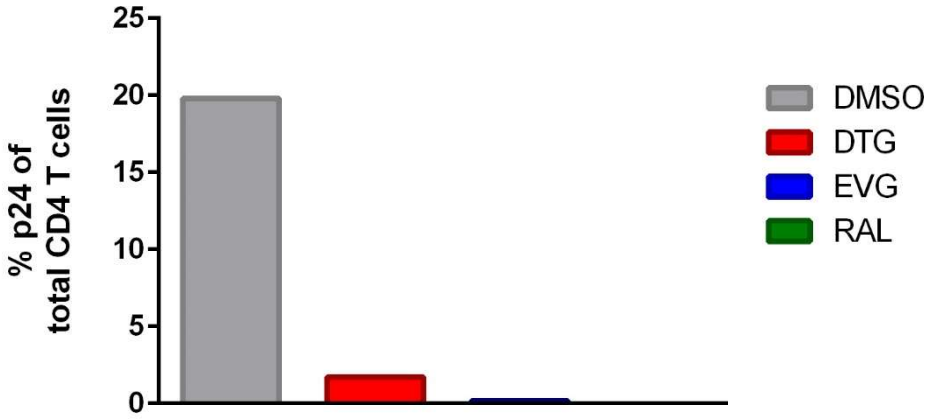

Figure 22: *In vitro* confirmation of biological antiviral activity of INSTI.

Comparison of HIV inhibitory activity of integrase inhibitors DTG, EVG and RAL showing that all three drugs were in their active form and able to inhibit HIV replication as measured by percentage of p24+ CD4 T cells from one donor via flow cytometry. Data indicate a one-time performance of the experiment to confirm inhibitory activity.

Our data collectively suggest that treatment with the newer INSTIs (DTG and EVG), had a significant negative impact on major CD8 T cell functions like cytokine expression, proliferation, T cell mobility, and the new synthesis of effector molecules as well as on the respiratory activity. The observed impact was not due to potential cellular cytotoxicity demonstrating stable viability over the same period of time.

3.2 Cytolytic CD4 T cells with follicular homing properties in chronic HIV infection

It has been demonstrated that the lymphoid B cell follicle is an important viral reservoir harboring high levels of HIV infection even when HIV is undetectable in the periphery. It is believed that the B cell follicle is an immune privileged site blocking HIV-specific CTL to enter, while T follicular helper cells can recirculate in and out of the follicle. Here, we describe a population of HIV-specific cytolytic CD4 T cells that have the ability to enter the B cell follicle and may be harnessed to reduce follicular viral burden.

3.2.1 Expanded subset of CD4+CXCR5+CD107a T cells in HIV-infected individuals

Given the current knowledge that HIV-specific CD8 T cells cannot enter the B cell follicle, we wanted to investigate whether a CD4 T cell population with follicular homing properties (CXCR5 expression) also have the ability to degranulate (CD107a expression) upon stimulation. Therefore, we isolated PBMCs from the blood of healthy donors, HIV+ treatment-naïve and HIV+ individuals on antiretroviral therapy. In addition, we isolated lymphatic mononuclear cells from secondary lymphoid tissues of healthy and HIV-infected individuals. To identify cytolytic CD4 T cells with follicular homing properties, we stimulated cells with SEB and distinguished CD4 T cell subsets between CXCR5+ CD107a- and CXCR5+ CD107a+ T cells using flow cytometry **(Figure 23)**.

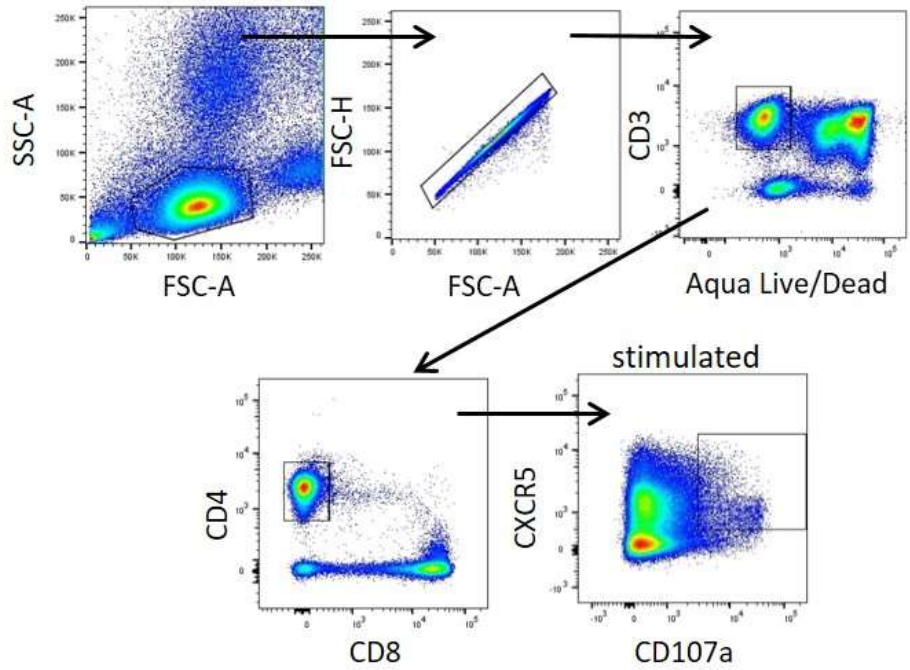

Figure 23: Gating strategy for cytolytic CD4 T cells with follicular homing properties.

Flow cytometry plots representing the gating strategy for cytolytic CD4 T cells with follicular homing properties for an HIV+ lymph node sample. After selecting all lymphocytes and single cells, live CD3 T cells were gated to distinguish between CD4 and CD8 T cells. Afterwards, CD4 T cells were distinguished between CXCR5+ CD107a- and CXCR5+ CD107a+ T cells. Gating was based on fluorescence minus one controls or for CD107a+ on an unstimulated negative control for each sample.

We could identify in the peripheral blood of healthy individuals, a CD4+ CXCR5+ T cell population (0.02±0.03) that showed the ability to degranulate and express CD107a

upon stimulation (from now on termed as T_{FC}). This cellular subset was significantly expanded in chronic HIV-infection of individuals without ART (0.24±0.23; p=0.0069) as well as in treated individuals (0.18±0.12; p=0.0063). In secondary lymphoid tissue, we could identify a much higher frequency of T_{FC} compared to the blood. This subset was also significantly (p=0.0317) expanded in chronic HIV infection (2.54±0.36) compared to healthy controls (1.60±0.48) (**Figure 24**).

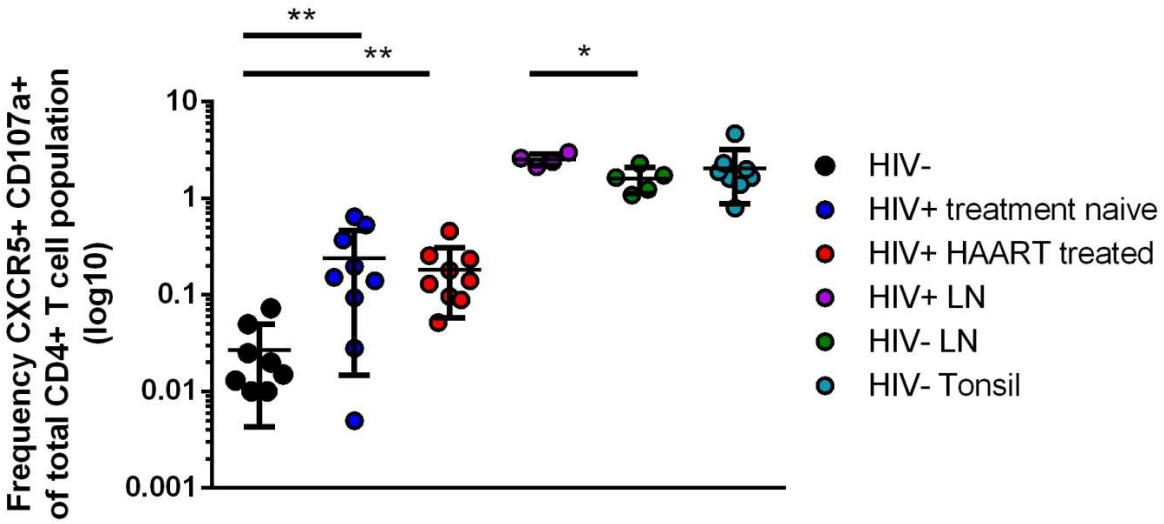

Figure 24: Frequency of CD4+CXCR5+CD107a T cells in the blood and secondary lymphoid tissue.

PBMCs from the blood and cells from different secondary lymphoid tissues were stimulated with SEB for 6 h and stained for CXCR5 and CD107a. We identified a CD4+ CXCR5+ T cell population that showed the ability to degranulate (CD107a) upon stimulation and were significantly increased in the blood of chronic HIV-infected individuals with and without ART as well as in lymphoid tissue of HIV+ individuals. The Chart shows the mean value ± SD. Statistical significance was assessed by RM one-way ANOVA test with Holm-Sidak´s multiple comparison test. (*P<0.05; **P<0.01; ***P<0.001).

Taken together, our data demonstrate a novel subset of cytolytic CD4+ T cells with follicular homing properties that can be found in the blood of HIV+ and HIV negative individuals and also more importantly in much higher frequency in secondary lymphoid tissue.

3.2.2 T_{FC} express high levels of CTLA-4 in the blood and lymph nodes

Next, we wanted to characterize the novel subset of cytolytic CD4+ T cells with follicular homing properties and compare this subset to CD4 T cells without homing receptor. We therefore determined via flow cytometry the expression of the exhaustion marker CTLA-4 for T_{FC} and compared this to the expression levels on CD4 CXCR5- T cells in

the blood and secondary lymphoid tissue in HIV+ and HIV negative individuals. CTLA-4 was expressed on CD4 T cells without CXCR5 in similar extend in the blood of HIV+ ART treated and HIV+ treatment-naïve individuals as well as in the lymph node of HIV+ and HIV negative individuals (2.70±1.12; 2.92±1.86; 2.75±1.25; 3.26±1.68, respectively). Interestingly, the expression of CTLA-4 was overall increased (7.29±1.41) for CD4 T cells in tonsil tissue of HIV negative individuals compared to blood and lymph node tissue. For CD4 T cells co-expressing CXCR5 and CD107a we found a significant increased expression of CTLA-4 in the blood of HIV+ patients, treatment-naïve and treated as well as in the lymph node of HIV+ and HIV negative individuals (9.24±2.06, p<0.0001; 8.69±4.07 p=0.0003; 12.89±1.24, p=0.0286; 12.37±1.71, p=0.0079; respectively). In tonsil tissue of HIV negative individuals, there was no significant increase (8.35±0.74) in the expression levels of CTLA-4 compared to CD4 T cells without homing receptor (**Figure 25**).

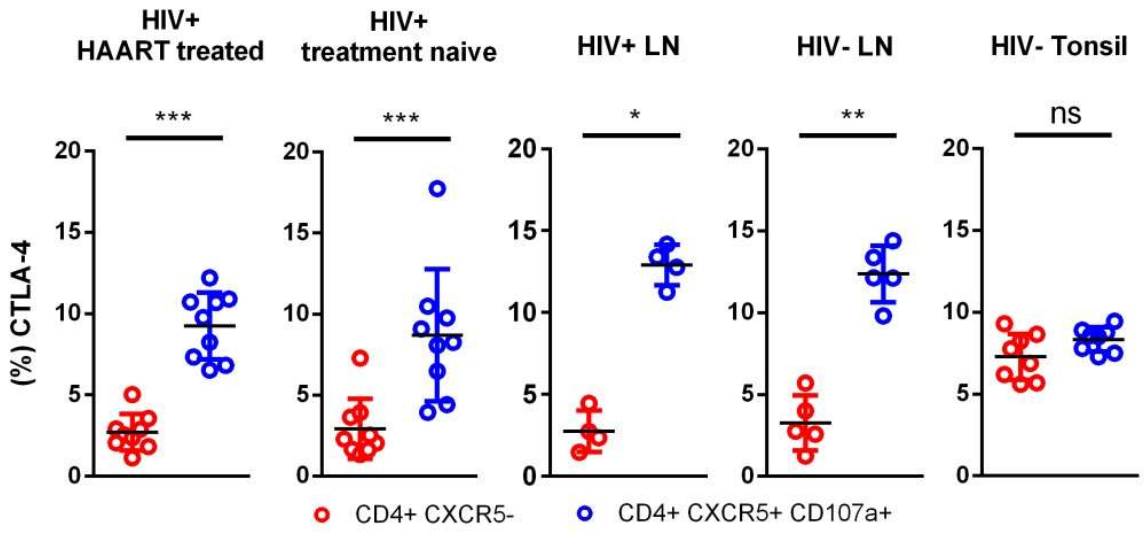

Figure 25: Expression levels of CTLA-4 for CD4 T cells co-expressing CXCR5 and CD107a.

The expression levels of CTLA-4 for CD4+ CXCR5- (red) were significantly decreased compared to CD4+ CXCR5+ CD107a+ (blue) T cells in blood samples from HIV+ HAART treated and treatment naïve patients, as well as in lymph node samples from HIV+ and HIV- individuals. The Chart shows the mean value ± SD. Statistical significance was assessed by student t test with Mann-Whitney test. (*P<0.05; **P<0.01; ***P<0.001).

Taken together, our data suggest an increased expression of CTLA-4 for CD4 T cells co-expressing CXCR5 and CD107a in the blood and lymph nodes, but not in tonsil tissue.

3.2.3 T_{FC} express high levels of PD-1 and show a T_{FH} phenotype

Given the increased expression of CTLA-4 in T_{FC}, we next wanted to see if this subset had higher expression of PD-1, what is not only an exhaustion marker but also an important marker to characterize T follicular helper cells especially in secondary lymphoid organs. We therefore determined via flow cytometry the expression of PD-1 in T_{FC} and CD4 T cells without follicular homing receptor and analyzed whether T_{FC} have a T_{FH} phenotype by co-expression of CXCR5 and PD-1. CD4 T cells without CXCR5 expression showed a similar low expression of PD-1 with a slight increase in the lymph nodes of HIV-infected individuals (9.24±2.79) and tonsils of HIV negative individuals (10.87±3.39) compared to the blood of HIV+ treated and treatment-naive patients as well as the lymph nodes of HIV negative individuals (3.94.87±3.81; 4.78±3.02; 5.37±2.22; respectively). However, T_{FC} showed a significantly higher expression of PD-1, although this increase was only seen in the lymphoid tissue but not in the blood. PD-1 expression levels for T_{FC} were significantly increased in the lymph nodes of HIV-infected individuals as well as in lymph nodes and tonsils of HIV negative individuals compared to CD4 T cells without homing receptor (62.99±19.92, p=0.0286; 31.27±14.94, p=0.0079; 58.17±9.02, p=0.0002; respectively) (**Figure 26**).

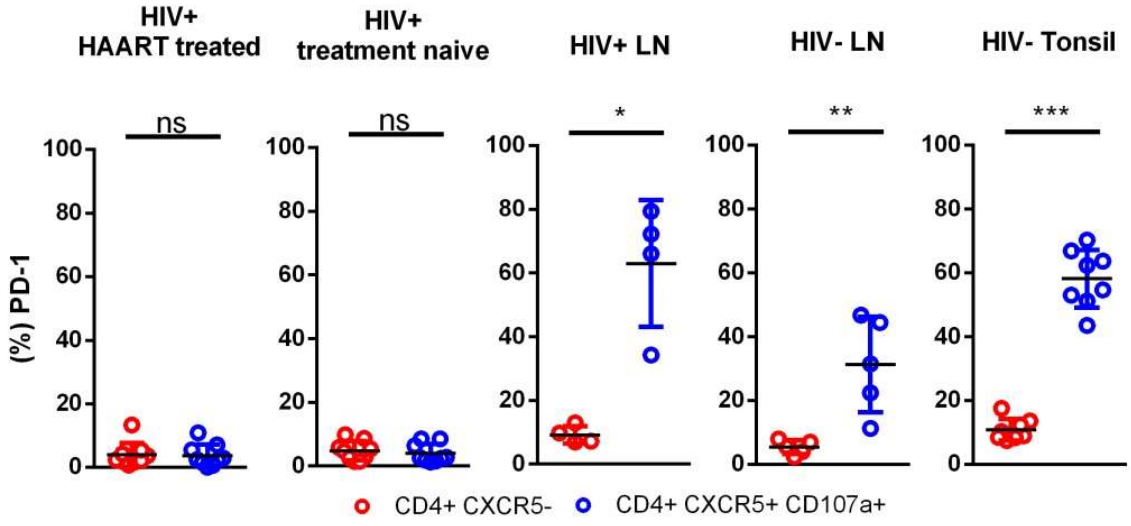

Figure 26: Expression levels of PD-1 for CD4 T cells co-expressing CXCR5 and CD107a.

The expression levels of PD-1 for CD4+ CXCR5- (red) were significant decreased compared to CD4+ CXCR5+ CD107a+ (blue). This difference was only seen in the lymphoid tissue but not in the blood. The Chart shows the mean value ± SD. Statistical significance was assessed by student t test with Mann-Whitney test. (*P<0.05; **P<0.01; ***P<0.001).

After we identified higher PD-1 expression for T_{FC} only in secondary lymphoid organs, we wanted to investigate whether this subset has not only cytolytic potential but also a T_{FH} phenotype. We performed an overlay analysis based on flow cytometry data and analyzed in what extend CD4 CD107a+ T cells also co-express CXCR5 and PD-1. Strikingly, CD4 CD107a+ T cells express CXCR5 and PD-1 together in a high extend, especially in tonsil tissue of HIV negative individuals. That was not the case for total CD4 T cells (**Figure 27**).

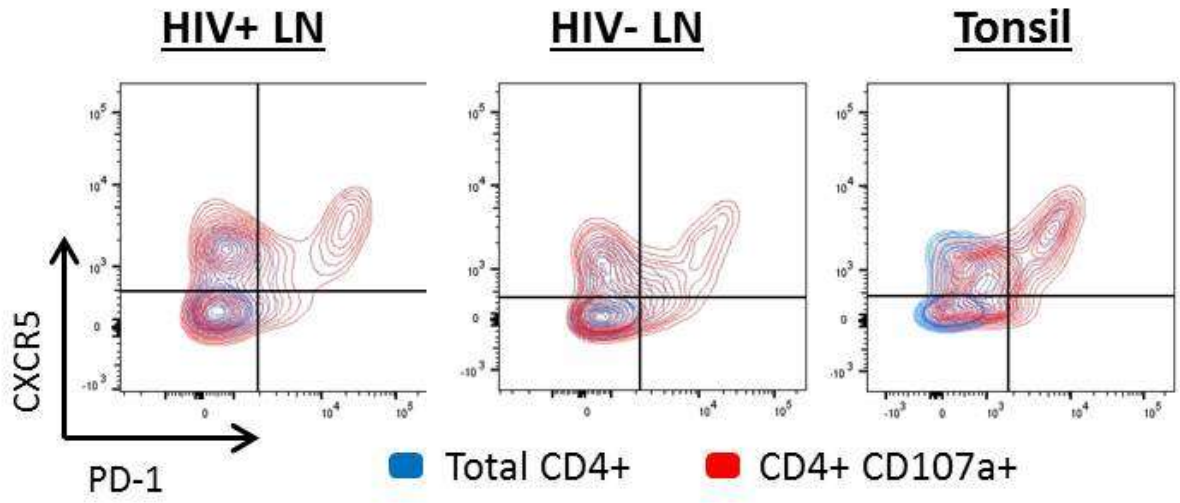

Figure 27: CD4 CD107a+ T cells in the lymphoid tissue showed a T_{FH} phenotype by co-expression of CXCR5 and PD-1.

Overlay analysis based on flow cytometry data identified CD4 CD107a+ T cells (red), which co-express CXCR5 and PD-1. Data were analyzed with FlowJo (Version 10.1).

In summary, our data have shown a significantly increased expression of PD-1 only for T_{FC} in lymphoid tissue and showed a T_{FH} phenotype.

3.2.4 Significant differences in the distribution of granzyme B and perforin expression between blood and secondary lymphoid tissue for T_{FC}

Given the expression of CD107a as a functional marker for identifying cytolytic activity, we next wanted to determine possible differences in the expression of specific cytolytic molecules. Therefore, PBMCs and mononuclear cells from lymphatic tissue were stimulated with SEB for six hours and afterwards analyzed the expression of granzyme B, perforin and IFNγ for T_{FC} and CD4 T cells without the homing receptor CXCR5. The expression of granzyme B and perforin differ between blood and lymphatic tissue. Expression of these two cytolytic molecules was elevated in CD4 T cells without

CXCR5 in the blood of HIV+ treated and treatment-naïve individuals compared to lymph nodes and tonsils from HIV-infected and non-infected individuals. Interestingly, the expression of granzyme B and perforin significantly increased for T_{FC} only in the lymph nodes and tonsils of HIV+ and HIV negative individuals compared to CD4 CXCR5- T cells (p=0.0286, p=0.0079, p=0.0002; p=0.05, 0.0079, p=0.0002; respectively). In fact, granzyme B expression was significantly decreased in T_{FC} of HIV-infected treated individuals compared to CD4 T cells without CXCR5 (p<0.0001) (**Figure 28**).

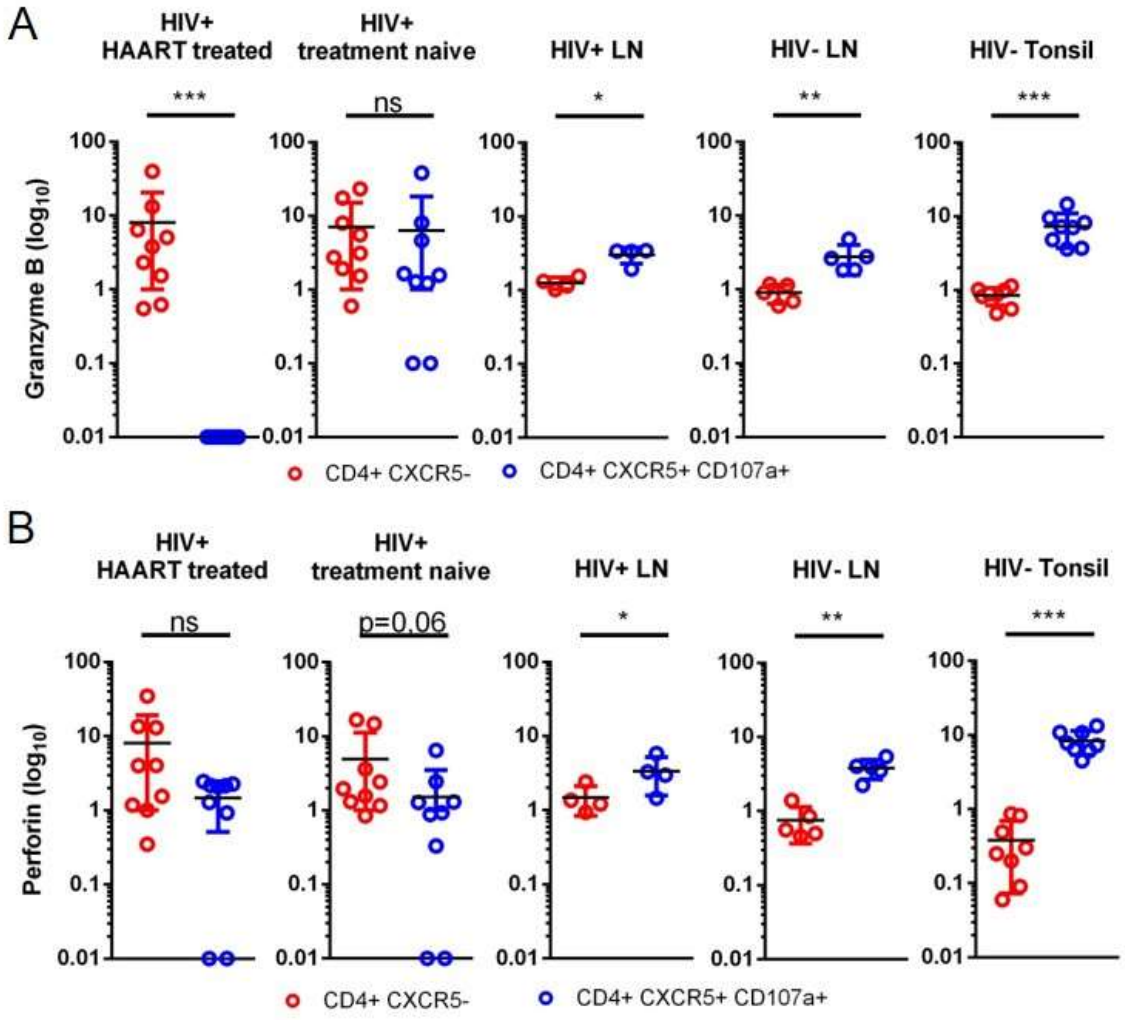

Figure 28: The expression of granzyme B and perforin differ between blood and lymphatic tissue.

PBMCs and cells from lymphatic tissue were stimulated with SEB for 6 hours and afterwards analyzed the expression of (A) granzyme B and (B) perforin for T_{FC} and CD4 T cells without the homing receptor CXCR5. CD4 CXCR5+ CD107a+ T cells (blue) in lymph nodes and tonsils expressed significantly higher levels of granzyme B and perforin compared to CD4 without CXCR5 (red). The chart shows the mean value ± SD. Statistical significance was assessed by student t test with Mann-Whitney test. (*P<0.05; **P<0.01; ***P<0.001).

After we identified significant differences in the expression levels of cytolytic molecules between blood and lymphatic tissue, we next wanted to analyze whether there are also differences for T_{FC} within the group of secondary lymphoid organs. The expression of both cytolytic molecules differed not between lymph nodes of HIV-infected and non-infected individuals. Surprisingly, expression levels of granzyme B and perforin for T_{FC} were significantly increased in tonsil tissue of HIV negative individuals compared to lymph node tissue of HIV+ and HIV negative individuals (7.31±3.61; 3.03±0.74, p=0.0390; 2.78±1.21, 0.0286; 8.36±2.98; 3.38±1.79, p=0.0140; 3.83±1.14 p=0.0290; respectively) (**Figure 29**).

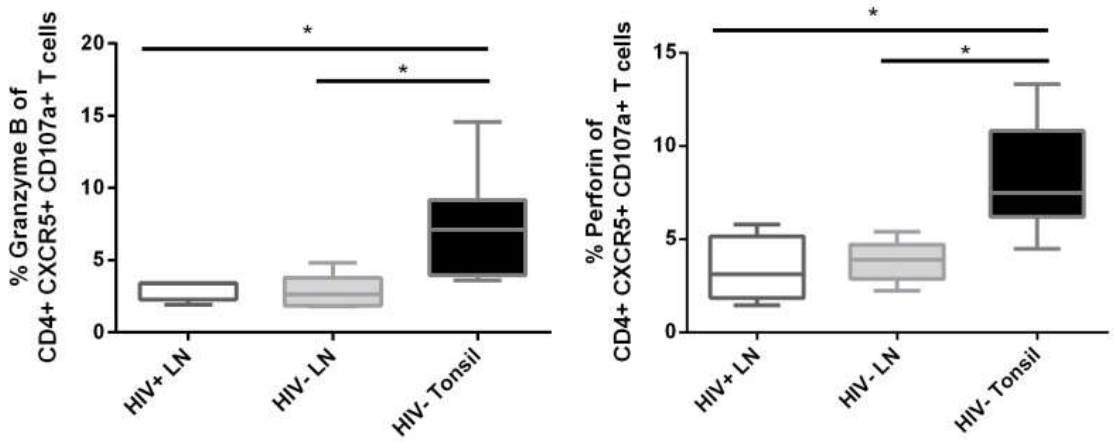

Figure 29: Significant differences in the distribution of granzyme B and perforin expression between the lymphatic tissues.

CD4 CXCR5+ CD107a+ T cells in tonsils expressed a significantly higher amounts of granzyme B and perforin compared to HIV+ and HIV- lymph nodes. The chart shows the mean value ± SD. Statistical significance was assessed by RM one-way ANOVA test with Holm-Sidak´s multiple comparison test. (*P<0.05; **P<0.01; ***P<0.001).

Given the differences in the expression of granzyme B and perforin between the lymphatic tissues, we analyzed the cytokine expression of the effector molecule IFNγ known to be a CTL-secreted soluble mediator. The frequency of IFNγ secreting CD4 T cells without CXCR5 was small and did not differ between lymph nodes of HIV-infected and non-infected individuals as well as tonsils of HIV negative individuals (3.83±0.68; 4.63±1.99; 2.67±0.95; respectively). In contrast, T_{FC} express significantly higher amount of IFNγ in all three lymphatic tissues (11.98±4.61, p=0.0286; 13.87±5.23, p=0.0079; 6.76±1.47 p=0.0002) (**Figure 30**).

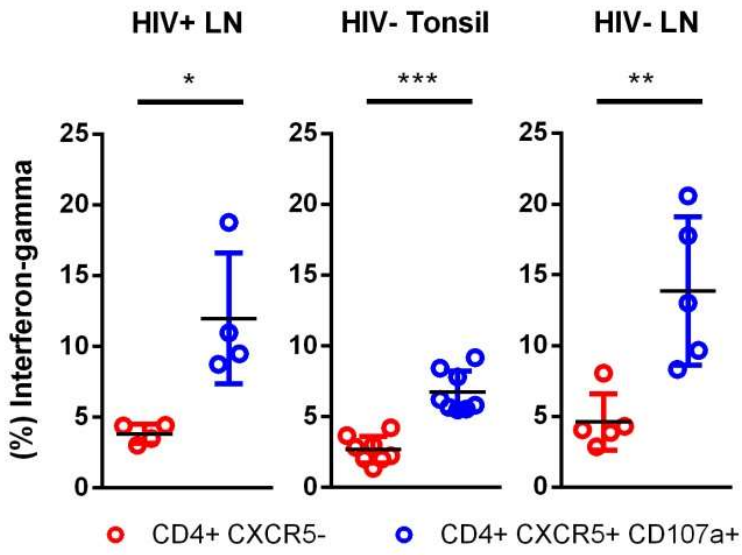

Figure 30: Significantly higher expression of IFNγ in T_{FC}.

Cells from lymphatic tissue were stimulated with SEB for 6 hours and afterwards analyzed the expression of IFNγ. CD4 CXCR5+ CD107a+ T cells (blue) in lymph nodes and tonsils expressed significantly higher levels of IFNγ compared to CD4 without CXCR5 (red). The chart shows the mean value ± SD. Statistical significance was assessed by student t test with Mann-Whitney test. (*P<0.05; **P<0.01; ***P<0.001).

Our data collectively demonstrated a novel subset of cytolytic CD4+ T cells with follicular homing properties, which expressed significantly higher levels of IFNγ as well as PD-1 and showed a T_{FH} phenotype. Moreover, we demonstrated that this subset differs in the distribution of granzyme B and perforin expression between blood, lymph nodes and tonsils.

4 Discussion

4.1 Impact of antiretroviral therapy regiments on CD8 T cell activity and function

Although different studies demonstrated that HIV-specific CD8 T cells play an important role in the control of viral replication and have the ability to suppress viremia to undetectable levels in elite controllers, they are not capable to eradicate the HIV reservoir even in the presence of highly efficient ART (38, 163, 164). HIV persists dormant in a small pool of infected cells and because these cells do not produce viral gene products, they remain invisible to immune surveillance (140, 165-167). Nonetheless, even through the use of small molecule latency reversing agents to activate virus transcription and viral protein production, HIV-specific CD8 T cells are unable to reduce the pool of latently infected cells (168). While the reasons for this are most likely multifactorial, we assessed in the present study the impact of ARVs on the function, proliferation, metabolism, migratory properties and inhibitory activity of CD8 T cells. We observed that treatment with the newer INSTIs (DTG and EVG) had a significant negative impact on major CD8 T cell functions as well as on the respiratory activity. We demonstrated that DTG, as part of first-line ART regimen, significantly decreased the ability of CD8 T cells to proliferate, migrate and express functional cytokines as well as reduced respiratory activity and more importantly the new-synthesis of cytolytic molecules. Our data suggest that the choice of ART could affect the current eradication strategies.

It is known that ART can partial restore HIV-specific CD8 T cell function, including a restored functional and less dysfunctional phenotype. Chronic HIV-infection is characterized by a progressive loss of CD8 T cell immune function. Important function like the capacity to simultaneously produce antiviral cytokines and release lytic molecules following antigenic stimulation is linked to a dysfunctional phenotype of HIV-specific CD8 T cells (163, 169). Because the immune response of these cells is highly dynamic and HIV-specific CD8 T cells exert selection pressure on HIV with the appearance of various escape mutations, it is important that new CD8 T cell responses are constantly emerging (133, 134, 170, 171). The increasing loss of function in CD8 T cells is associated with the upregulation of immune checkpoint markers and progressive exhaustion of T cells. Studies have shown that ART can partially prevent the increasing loss of function in CD8 T cells and preserve a functional phenotype

(172). However, despite a partial improved function, HIV-specific CD8 T cells on ART show signs of overall dysfunction in comparison to HIV-uninfected individuals. Studies have demonstrated a heightened immunosenescence under ART compared to HIV negative individuals. Importantly, several studies have demonstrated that HIV-specific CD8 T cells from ART-treated individuals with suppressed viremia do not exhibit the same breadth of function as HIV-specific CD8 T cells from HIV elite controllers. This includes a reduced proliferation capacity and the ability to express cytokines as well as cytolytic molecules upon stimulation with HIV antigens (140, 173). In comparison to elite controllers, HIV-specific CD8 T cells from ART-suppressed individuals show as well a reduced capacity to eliminate both; productively and latently infected CD4 T cells (174, 175). One of the prominent findings in the last years is the increased activation of latently infected cells through shock and kill strategies. Although these cells get reactivated, several clinical trials showed so far only limited or no clearance (158-160). It is surprising that these approaches have no impact on the HIV latent reservoir pool indicating a lack of CD8 T cells' ability to inhibit viral replication.

For this study, we assessed the impact of ARV classes on the function of CD8 T cells. In both *ex vivo* and *in vitro* experiments, we demonstrated that a treatment with INSTIs, especially DTG, decreased CD8 T cells' ability to proliferate and secrete effector cytokines. In particular, in our *ex vivo* experiments, we demonstrated that individuals on an INSTI-based ART regimen had a measurable reduction of their multifunctional CD8 T cell response. While CD8 T cell polyfunctionality has been linked to the inhibitory capacity of CD8 T cells, we were also able to demonstrate that the ART choice affected the production of cytolytic molecules, T cell proliferation and migratory capacity of CD8 T cells. This data suggest that the choice of ARVs can have an important impact on numerous functions of CD8 T cells and may influence the success of "shock and kill" strategies. Previous studies have already highlighted several explanations for the failure of LRAs to reduce the viral reservoir. The B cell follicle structure promotes the possibility for HIV to hide and not been detected by effector cells like CD8 T cells. This creates an immune privileged site and activation with LRAs, especially histone deacetylase inhibitors (HDACi), may only have a limited impact as activated virus-producing cells may not be reached by virus-specific CD8 T cells due to compartmentalization (176, 177). This compartmentalization in lymphoid organs could prevent effective killing even after successful activation of latently infected cells and demolish the clearance of the HIV reservoir. Additionally, studies showed that

LRAs also may have a cytotoxic effect on immune cells. It has been demonstrated that the HDAC inhibitors, romidepsin and panobinostat, have highly cytotoxic effects on CD8 T cells (178, 179). While both are possible explanations, the reason for failed shock and kill attempts Is most likely multifunctional and this data may provide an additional element. We could demonstrate that treatment with the newer INSTIs significantly decreased proliferation, migration and the expression of effector molecules simultaneously. All these functions are important when it comes to the effective killing of infected cells as well as to the control of the infection. This suggests that the choice of INSTI for treatment before shock and kill therapies may not be the best. In addition, INSTI as well as NRTI/NNRTI block HIV entry into the genome and therefore the presentation of any viral gene products. In contrast, a double PI regimen can boost the production of new viral RNA and the expression of uncleaved viral protein precursors. With this ART regimen would be a strategy during shock and kill trials, LRAs are able to activate viral transcription in the absence of fully viral assembly and latently infected CD4 T cells can be recognized and killed by effector cells.

Furthermore, we observed a significant impairment of mitochondrial respiration of CD8 T cells after exposure to the integrase inhibitor DTG. This could be a possible explanation for the decreased function of CD8 T cells. Moreover, we previously published that the same ARVs from the INSTI class, DTG and EVG, had a similar impact on the CD4 T cells respiratory activity compared to CD8 T cells. It was shown that both INSTIs interact with the electron transport chain of mitochondria and severely impairing the respiratory capacity of CD4 T cells. Additionally, increased production of reactive oxygen species (ROS) as well as higher content of mitochondrial DNA were linked to a treatment with INSTI. These changes led to a skewing of CD4 T cell immune response toward a reduced and monofunctional CD4 T cell response (82). It has been reported that INSTI treatment, especially DTG- and EVG-based regimens, are linked to specific side effects like weight gain and metabolic disorders (180). Recent studies highlighted a possible role of DTG in body fat composition changes in individuals who switched from efavirenz-based to DTG-based regimens (181). In addition, Moure et al. reported elevated levels of the antidiabetic hormone fibroblast growth factor 21 (FGF21) in human hepatocytes after treatment with EVG. Higher levels of FGF21 is generally associated with insulin resistance and glycaemia (182). Although we did not see any impact on cellular glycolysis, the reported side effects of an INSTI treatment points to a deteriorated and disrupted mitochondrial function. Especially significant

increased weight gain after switching to DTG-based regimens can be linked to impaired reduced oxygen consumption in obese individuals (183, 184).

This study's limitations are the determination of the precise mechanism of action of INSTI on CD8 T cell activity, which needs further investigation. It is conceivable that DTG and EVG interfere in a very similar way with the electron transport chain of mitochondria compared to CD4 T cells and further experiments are needed to determine the interaction partner (e.g. redox carrier) inside the transport chain.

4.2 Cytolytic CD4 T cells with follicular homing properties in chronic HIV infection

Over the last years' progress has been made to identify that HIV preferentially targets T_{FH} cells in B-cell follicles of secondary lymphoid organs for both; long-term latent infection and the persistent production of infectious viral particles (185-187). Cytolytic cells, like the majority of HIV-specific CD8 T cells, are excluded from B-cell follicles which makes the B cell follicle an immune privileged site blocking effector cells to enter (177, 188, 189). In contrast, it is known that T follicular helper cells can recirculate in and out of the follicle, mainly through the expression of the chemokine receptor CXCR5 (190). However, here we identified a CD4+ CXCR5+ T cell population that showed the ability to degranulate (CD107a) upon stimulation and was significantly expanded in the blood and lymph nodes of chronic HIV infected individuals. We observed that upon stimulation, this subset expressed not only higher levels of CTLA-4 as well as PD-1 compared to bulk CD4 T cells, but also showed a T_{FH} phenotype. More interestingly, we showed that this subset expresses higher amounts of cytolytic molecules in lymph nodes of HIV infected individuals compared to bulk CD4 T cells.

In comparison to other subsets of CD4 T cells, like Tregs, T_{FH} cells have the unique characterization to express CXCR5 on their surface. This not only phenotypically distinguishes T_{FH} cells from these other subsets but functionally drives T_{FH} cell migration into B cell follicles in a CXCL13 dependent manner (114, 191, 192). It is known that the transcription factor BCL-6 is the main driver for the differentiation of T_{FH} cells, although recent studies demonstrated in mice that CXCR5 appears to be expressed in a BCL6-independent manner in the early stage of differentiation (193, 194). The differentiation of cytolytic CD4 T cells on the other hand is driven through

the interplay of the transcription factors EOMES and T-bet (125). A recent publication identified EOMES+ CD4 T cells within the lymph node, which expressed effector molecules and had greatly reduced T-bet expression. This observation suggests that EOMES may play a more important role for the differentiation of CTL CD4 T cells in secondary lymphoid organs. Our data show that the majority of cytolytic CD4 T cells in the lymph nodes also co-express CXCR5 as well as PD-1 and have a T_{FH} phenotype. Although PD-1 is mainly known as an exhaustion marker of T cells in the blood, T_{FH} cells also express high levels of this marker and is therefore used to characterize T_{FH} cells (195). Our data support the hypothesis of an early differentiation to T_{FH} cells and second subsequent expansion to CTL CD4 T cells, possibly through the upregulation of EOMES. Further experiments are needed to determine any change of transcription factors and to identify under which cytolytic properties are induced.

It is known that cytolytic CD4 T cells contribute to the control of infection progression and HIV-specific cytolytic CD4 T cells emerge early during acute HIV infection and are associated with early viral control (124, 125). Despite that, previous studies on CD4 T cells are mostly focused on function, phenotype and transcriptional characteristics in HIV infection from peripheral blood T cells. It has been shown that secondary lymphoid tissues harbor CD4 T cells with different plasticity and functional characteristics, then their counterpart in the blood (196). Our data contribute to these findings. We found a subset of CD4 T cells with follicular homing properties and degranulate upon stimulation as well as express effector molecules like perforin or granzyme B. This subset was significantly higher expressed in lymph nodes compared to the blood of HIV-infected individuals. Effector CD4 T cells in the lymph nodes can play a major role in clearing the HIV reservoir due to the fact that B cell follicles are an immune privilege site. The majority of CD8 T cells do not express CXCR5 and cannot enter the B cell follicle. Although recent studies localized CXCR5 expressing CD8 T cells within the B cell follicle. It was shown that CD8 T cells in HIV-infected LNs generally express low-levels of cytolytic molecules (197-199). Our data on cytolytic CD4 T cells with follicular homing receptors can already show increased expression levels of effector molecules. Through therapeutic intervention, which harnesses this cellular subset, it can may provide a novel strategy for HIV eradication attempts in the future.

Nevertheless, limitation of this study is the determination of the viral inhibitory activity through the cytolytic molecules perforin and granzyme B. Buggert et al. described

previously a general lack of effector functions and cytolytic capacity of total CD4 T cells in lymph nodes of HIV-infected individuals (200). These results are inconsistent with ours and need further investigation.

5 Summary

HIV-specific cytolytic T cells play an important role in the control of viral replication and have the ability to suppress viremia to undetectable levels in HIV elite controllers, but they are not capable to eradicate the HIV reservoir even in the presence of highly efficient ART. It is known that cytolytic cells, like HIV-specific CD8 T cells, are excluded from B-cell follicles which makes the B cell follicle an immune privileged site blocking effector cells to enter.

HIV-specific CD8 T cells fail to clear infection even in the presence of ART and cure strategies such as “shock-and-kill”. Little is known how ART contributes to HIV-specific CD8 T cell function and the ability to clear HIV infection. Here, the impact of different ART regimens on the function of CD8 T cells were assessed to proliferate, migrate and express effector cytokines.We observed that treatment with the newer INSTIs (DTG and EVG) had a significant negative impact on major CD8 T cell functions as well as on the respiratory activity. We demonstrated that DTG, as part of first-line ART regimen, significantly decreased the ability of CD8 T cells to proliferate, migrate and express functional cytokines as well as reduced respiratory activity and more importantly the new-synthesis of cytolytic molecules. Our data collectively suggest that treatment with the newer INSTIs (DTG and EVG), The observed impact was not due to potential cellular cytotoxicity demonstrating stable viability over the same period of time. Our data suggest that the choice of ART could affect the current eradication strategies.

It is known that T follicular helper cells can recirculate in and out of the follicle, mainly through the expression of the chemokine receptor CXCR5, where cytolytic cells cannot enter. Here, we describe a population of HIV-specific cytolytic CD4 T cells that have the ability to enter the B cell follicle. We identified a CD4+ CXCR5+ T cell population that showed the ability to degranulate (CD107a) upon stimulation and was significantly expanded in the blood and lymph nodes of chronic HIV infected individuals. We observed that upon stimulation, this subset expressed not only higher levels of CTLA-4 as well as PD-1 compared to bulk CD4 T cells, but also showed a T_{FH} phenotype. More interestingly, we showed that this subset expresses higher amounts of cytolytic molecules in lymph nodes of HIV infected individuals compared to bulk CD4 T cells. This subset may be harnessed to reduce follicular viral burden in the reservoir.

Zusammenfassung

HIV-spezifische zytolytische T-Zellen spielen eine wichtige Rolle bei der Kontrolle der Virusreplikation und haben die Fähigkeit die Viruslast in HIV-Elite-Controllern auf eine nicht nachweisbare Grenze zu reduzieren. Sie sind allerdings nicht in der Lage, das HIV-Reservoir selbst in Gegenwart von hocheffizienter antiretroviraler Therapie (ART) zu eliminieren. Es ist bekannt, dass Effektorzellen wie HIV-spezifische CD8-T-Zellen von B-Zell-Follikeln ausgeschlossen sind, was das Follikel zu einer immunprivilegierten Stelle macht, die den Eintritt von Effektorzellen blockiert.

Es ist allerdings wenig bekannt, wie ART zur HIV-spezifischen CD8-T-Zellfunktion und zur Fähigkeit der Eliminierung der HIV-Infektion zum Beispiel während einer „Shock and kill“ Strategie beiträgt. Hier wurde der Einfluss verschiedener ART-Kombinationstherapien auf die Funktion von CD8-T-Zellen zur Proliferation, Migration und Expression von Effektorzytokinen hin untersucht. Wir beobachteten, dass die Behandlung mit den neueren INSTIs (DTG und EVG) einen signifikanten negativen Einfluss auf die Hauptfunktionen der CD8-T-Zellen, sowie auf die metabolische Aktivität hatte. Wir haben gezeigt, dass DTG als Teil der ART-Kombinationstherapie der ersten Wahl die Fähigkeit von CD8-T-Zellen; sich zu vermehren, zu migrieren, funktionelle Zytokine zu exprimieren, sowie die metabolische Aktivität und vor allem die Neusynthese von zytolytischen Molekülen signifikant verringert Der beobachtete Einfluss war nicht auf eine mögliche zelluläre Zytotoxizität zurückzuführen. Unsere Daten deuten darauf hin, dass die Wahl von ART die aktuellen Eliminierungsstrategien von HIV beeinflussen könnte.

Es ist bekannt, dass T-follikuläre Helferzellen hauptsächlich durch die Expression des Chemokinrezeptors CXCR5 in das Follikel und aus diesem heraus zirkulieren können, wodurch zytolytische Zellen der Eintritt verwehrt bleibt. Hier beschreiben wir eine Population von HIV-spezifischen zytolytischen CD4-T-Zellen, die in das B-Zell-Follikel eindringen können. Wir identifizierten eine CD4 + CXCR5 + T-Zellpopulation, die die Fähigkeit zur Degranulation (CD107a) bei Stimulation zeigte und sowohl im Blut, als auch in den Lymphknoten chronisch HIV-infizierter Personen signifikant erhöht war. Wir beobachteten, dass diese Untergruppe bei Stimulation nicht nur höhere CTLA-4- und PD-1-Levels im Vergleich zu CD4-T-Bulk-Zellen exprimierte, sondern ebenfalls einen T_{FH}-Phänotyp zeigte. Wir konnten darüber hinaus bestimmen, dass diese

Untergruppe im Vergleich zu CD4-T-Bulk-Zellen höhere Mengen an zytolytischen Molekülen in Lymphknoten von HIV-infizierten Personen exprimierte. Diese Untergruppe könnte dafür genutzt werden, um die follikuläre Viruslast im Reservoir zu verringern.

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7 Abbreviations

3TC	Lamivudine
ABC	Abacavir
AIDS	Acquired immune deficiency syndrome
APC	Antigen-presenting cell
APV	Amprenavir
ART	Antiretroviral therapy
ATV	Atazanavir
AZT	Zidovudine
BCR	B cell receptor
bnAbs	Broadly neutralizing antibodies
CD4 CTL	Cytolytic CD4 T cells
CD40L	CD40 ligand
CDC	Centers for Disease Control and Prevention
CDR	Complementarity determining region
CFSE	Carboxyfluorescein succinimidyl ester
d4T	Stavudine
ddC	Zalcitabine
ddI	Didanosine
DLV	Delavirdine
DN	Double negative
DP	Double positive
DRV	Darunavir
DMSO	Dimethylsulfoxid
DTG	Dolutegravir

ECAR	Extracellular acidification rate
EFV	Efavirenz
EIA	Enzyme immunoassay
EOMES	T-box brain protein 2
ETV	Etravirine
EVG	Elvitegravir
FCCP	p-trifluormethoxy carbonyl cyanice phenol hydrazine
FCS	Fetal calf serum
FDA	Food and Drug Administration
FGF21	Fibroblast growth factor 21
FO	Follicular zone
FTC	Emtricitabine
HDACi	Histone deacetylase inhibitors
HIV	Human immunodeficiency virus
HPGM	Hematopoietic progenitor growth medium
HR	Heptad repeat
HSC	hematopoietic stem cell
HTLV-III/LAV	Human T-lymphotropic virus, type 3 / lymphadenopathy-associated virus
IDV	Indinavir
Ig	Immunoglobulin
IgH	Heavy chain of the immunoglobulins
IgL	Light chain of the immunoglobulins
LAMP	Lysosome-associated membrane glycoproteins
LPV	Lopinavir
LRA	Latency reversing agent

MAD	Mean accumulated distance
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MSM	Men who have sex with men
MZ	Marginal zone
NFV	Nelfinavir
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
NVP	Nevirapine
OCR	Oxygen consumption rate
PAMPS	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
PI	Protease inhibitor
PIC	Pre-integration complex
PWID	People who inject drugs
RAL	Raltegravir
RLP	Rilpivirine
ROS	Reactive oxygen species
RTV	Ritonavir
SEB	Staphylococcus entrotoxin B
T-bet	T-box transcription factor TBX21
TAF	Tenofovir alafenamide
TCR	T cell receptor
TDF	Tenofovir disoproxil fumarate
T_{FH}	T follicular helper cells

TPV	Tipranavir
Tregs	Regulatory T cells
TRL	Toll-like-receptor
UNAIDS	United Nations Program on HIV/AIDS

8 List of figures

9 List of tables

Statement: Previously published figures

In the context of this doctoral work, the **figures 21 and 22** were already published in the following article:

Effect of HIV infection and antiretroviral therapy on immune cellular functions. Korencak M, Byrne M, Richter E, Schultz BT, Juszczak P, Ake JA, Ganesan A, Okulicz JF, Robb ML, de Los Reyes B, Winning S, Fandrey J, Burgess TH, Esser S, Michael NL, Agan BK, Streeck H. JCI Insight. 2019 Jun 20;4(12)

Thereby all experiments for these figures were performed by myself.

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