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EXPERIMENTELLE VIROLOGIE**

**Characterization of regulatory T cells and Th17 cells
as reservoir of HIV-1 in chronic infection**

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Dedicated to my Grandfather.

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1. Introduction

The human immune deficiency virus (HIV) was identified in 1983 as the causative agent of the acquired immune deficiency syndrome (AIDS). In the decades that have passed since the initial discovery of the pathogen [1], a combination antiretroviral therapy has been developed through intensive research in the field, and the formerly lethal disease is now a chronic, manageable condition when treated [2–4]. A cure, however, remains an elusive goal. The high cost of life-long therapy restricts universal access to medication to developed countries, and in other parts of the world millions infected with the virus still remain untreated and die of complications of the disease [5,6].

Current state-of-the-art therapy targets various stages of the viral life cycle through the administration of a combination of different active compounds termed combined antiretroviral therapy (cART) [7,8]. The licensed forms of medication are highly effective at blocking viral entry, replication and integration into the human genome, as well as viral particle production, however they are powerless against the latent proviral form of HIV. In this form, the viral genome may persist for decades – a phenomenon which represents a major obstacle to a cure.

A suboptimal response by the host immune system is also an important contributor to the difficult nature of eliminating HIV infection. Even though both innate and adaptive immune responses are mounted against the virus [9], they are ineffective at preventing the development of a latent viral reservoir and clearing the pathogen. An ongoing state of immune activation, even in the case of effective treatment, is a hallmark of HIV disease, and is responsible for some of the pathology associated with chronic infection [9,10].

Even though established HIV infection cannot be eliminated to our current knowledge, a small minority of HIV patients, termed “elite controllers” (EC) [11], are able to maintain a steady state of no viremia without any treatment for decades. The immunologic and virologic mechanisms behind this phenomenon remain incompletely under-

stood. These patients represent the model for a so-called “functional cure” [12], a state in which a subject is not considered free of the virus, but HIV is maintained indefinitely in its latent form without the need for therapy.

The term of “sterilizing cure” is used to describe a hypothetical scenario in which a patient becomes free of any proviral HIV that could give rise to a relapse of viremia. The only anecdotal case of such a cure is that of the “Berlin patient”, an HIV infected male who had received repeated myeloablative chemotherapy as well as full body irradiation for treatment of acute myelogenous leukemia and was then transplanted with donor hematopoietic stem-cells with an inherent resistance to HIV [13]. The patient maintains practically undetectable levels of all forms of HIV without treatment for many years, and thus, remains the only person to be considered cured of the virus [14].

Together with the momentum generated by the enormous success that the development of cART has been able to achieve in just 30 years, the above mentioned reports are continuing to fuel initiative in the field of HIV cure research. Any approach to eliminate latent HIV infection will require a deeper knowledge of the distribution of the virus in its proviral form among the different cellular reservoir sites that have been implicated in previous studies. The aim of this work was to contribute to our understanding of this topic.

1.1. HIV-1 infection

There are two phylogenetically different forms of HIV, named HIV-1 [1] and HIV-2 [15]. Both are members of the family of human retroviruses (*Retroviridae*), and the subfamily of lentiviruses, and they represent two of the four retroviruses known to cause human disease (the other two being Human T-cell Leukemia Virus type I and type II / HTLV-I and HTLV-II) [16]. The overwhelming majority of HIV infected people are infected with HIV-1, a virus that probably originates from chimpanzees and/or gorillas [17]. HIV-2 cases are mostly restricted to, or can be traced back to West Africa, and the virus most likely represents a separate transfer to humans from sooty mangabeys [18]. The remainder of this work will focus solely on HIV-1.

1.1.1. Epidemiological considerations

HIV-1 can be classified phylogenetically according to groups M (major), O (outlier), N and P. The natural reservoir for groups M and N are chimpanzees, while O and P can be traced back to Cameroonian gorillas. The majority of HIV infections today are caused by Group M (major) viruses, which can be subdivided into subtypes, also termed clades A – D and F – J, as well as so-called circulating recombinant forms (CRFs) that are created in co-infection and subsequent recombination between the respective clades.

Clade C viruses are mostly found in South- and East-Africa and India, and are the most common type, responsible for around 50% of all infections worldwide. Viruses that predominate in America, Europe and Australia belong to clade B [19]. Genetic variation within a subtype is in the range of 15-20%, while variation within subtypes is approximately 25-35% [19,20]. This genetic diversity is an important feature of HIV, which has implications for the manifestations of HIV disease as well as the immune response to infection.

As of the year 2014, 36.9 million people globally were infected with HIV, with more than two thirds of the affected population living in Africa. The number of new infections per year has been in a steady decline since 1998, with a 35% reduction in global incidence since the year 2000, when less than 1% of people in low income countries had access to therapy. In 2014 the global access to cART was 40%, meaning 22 million people worldwide were still not being treated for HIV. An estimated 17.1 million people were unaware of their infection [21].

HIV is a sexually transmitted disease (STD). By far the most common route of contracting HIV infection is through heterosexual transmission [21,22]. The overall efficiency of this transmission route is low. Risk factors that increase the chances of infection are a high viral load, the presence of ulcerative and inflammatory genital lesions, as well as co-existing STDs [22]. Unprotected receptive anal intercourse in both women and men (MSM – men who have sex with men) are associated with a higher risk of transmission [2].

HIV may also be transmitted parenterally by intravenous, intramuscular or subcutaneous injection [2,22]. Intravenous drug users may contract the virus by sharing injection paraphernalia, and remain a risk group to date. The chances of contracting HIV through contaminated blood and blood products in a hospital setting are extremely low, due to the screening of donors and donated blood, but it may still happen in low resource settings. Health care workers may be exposed to HIV through accidental needle injuries, or exposure of non-intact skin or mucous membranes to the bodily fluids of HIV patients [22].

1.1.2. The viral life cycle

The mature HIV virion is an icosahedral structure, consisting of a number of viral as well as host cell proteins and two copies of a full length, 9 kB - long viral RNA encoding the viral genome. The three viral genes *gag*, *pol* and *env* are common to all retrovirus-

es. The Gag protein products matrix (MA), capsid (CA), nucleocapsid (NC) and p6, together with the Env proteins surface (SU or gp120) and transmembrane (TM or gp41) make up the structure, while the Pol proteins protease (PR), reverse transcriptase (RT) and integrase (IN) are also packaged into the viral particle [23]. The rest of the HIV proteins (accessory and regulatory proteins) are each coded for by separate viral genes. Out of these, Vif, Vpr and Nef are also included in the virion, while the proteins Tat, Rev and Vpu are not [23].

HIV entry into the cell requires the attachment of the SU (gp120) with its receptor on the host cell, cluster of differentiation 4 (CD4), as well as the resulting conformational changes that allow for binding to one of the HIV co-receptors C-C chemokine receptor 5 (CCR5, also CD195) or the C-X-C chemokine receptor 4 (CXCR4, also CD184) [24]. CCR5 binds non-syncytium forming HIV isolates that have a tropism for macrophages (also termed R5 viruses), while CXCR4 binds syncytium inducing T-cell tropic viruses (X4 viruses) [23]. Transmission of HIV is mediated by R5 viruses, while X4 viruses dominate in late stage HIV infection [25]. The sequential conformational changes involving SU, CD4 and the co-receptors induces a conformational change in TM (gp41), leading to fusion of the viral and cellular membranes, and release of the virion core into the cytoplasm [23,24].

Viral entry is followed by two equally important steps in the viral life cycle, namely the process of uncoating and reverse transcription of the viral RNA genome to DNA in the reverse transcription complex (RTC). Whether these processes take place in a sequential order or in parallel is an area of debate [26,27].

The RTC comprises the two copies of viral RNA genome, tRNA₃^{Lys} primer, RT, IN, MA, NC, Vpr and various host proteins [28]. The viral protein Vif is also a part of the RTC, and it has an important role in counteracting cellular retroviral restriction factors APOBEC3G and TRIM5 α [29]. RT has two enzymatic activities that are necessary and sufficient to carry out the synthesis of the viral DNA genome: reverse transcriptase and RNase H. Minus and plus strands of a viral DNA genome are synthesized in a

sequential manner including two strand transfer events, while both copies of the RNA genome may serve as templates and are eventually degraded by the RNase H activity of the RT during the process [23,26,28]. A characteristic triple stranded central DNA flap remains as a remnant of the reverse transcription process [26,28,30]. RT is the target of anti HIV medication of the nucleotide – (NRTI) and non-nucleotide (NNRTI) reverse transcriptase inhibitor family. HIV RT does not have a proofreading mechanism, and as for, reverse transcription of HIV results in a relatively high mutation rate [28]. It should be acknowledged, that errors made during the host RNA-polymerase II mediated transcription of the HIV RNA genome [31] (see later) may also contribute to a smaller extent, and mutations may also be caused by the above mentioned APO-BEC3G retroviral restriction protein [29]. The combined effect of the above mentioned mechanisms results in an overall mutation rate of 2×10^{-5} errors per nucleotide per replication cycle [28].

The process of reverse transcription culminates in the generation of the pre-integration complex (PIC), which is composed of the double-stranded viral DNA, IN, MA, Vpr and RT, as well several host cell proteins. In contrast to other retroviruses, HIV is able to infect non-cycling cells, i.e. cells with an intact nuclear membrane [26,27,30]. Thus, for PIC trafficking, a mechanism involving various karyophiles must be in place to ensure entry into the nucleus. Such a role has been implicated for IN, MA and Vpr [27] as well as the above mentioned DNA flap on the viral cDNA [30].

Once inside the nucleus, integration into the host cell genome is initiated. Like RT, IN also possesses two enzymatic activities to facilitate this process. First, the viral double stranded cDNA is processed on the 3' end, where two nucleotides are clipped of each terminus with high specificity [32]. Second, IN must engage a sequence in the host chromatin for the proviral DNA to be integrated, called the strand transfer activity. The host DNA is cut, and the free 3' ends of the viral DNA are joined to the free 5' phosphates of the chromosomal DNA. The enzyme specificity for acceptor site sequences at this second step is quite limited, but statistically significant: HIV integration

preferentially takes place in the introns of actively transcribed genes [32–34]. Host cell gap repair of the DNA recombination intermediate ensures the integrity of the genome, and produces a characteristic 5 base pair (bp) duplication of target DNA sequences flanking the proviral genome. The dependency of the HIV life cycle on the viral IN is exploited by a new class of drugs named integrase inhibitors [35,36].

Following integration of the proviral DNA into the host genome, the infection of the cell is complete. The fate of the infected cell may then theoretically take three directions: 1.) abortive infection resulting from integration of a defective provirus 2.) productive infection resulting in infectious particle release 3.) latent infection (the cell may revert to a state of latent infection after productive infection). Points 1 and 2 will be discussed here, while latent infection is covered in a later section of this work.

1.1.1.1. Abortive infection

The high mutation rate of HIV, resulting in a characteristic genetic heterogeneity was already mentioned in previous sections. If the integrating proviral DNA acquires inactivating mutations that make normal transcription, translation and/or assembly of the infectious particle impossible, then the infectious cycle is terminated at this point, and the integrated defective proviral DNA is termed replication-incompetent. The most common inactivating mutations are large internal deletions and APOBEC3G mediated hypermutations resulting in premature stop codons [37]. In chronic HIV infection of patients treated with cART, replication incompetent proviral DNA sequences are estimated to be 300 times more common, than those encoding for replication competent virus [37,38].

The pathophysiological role of defective integrated proviruses is not well understood, and their significance is a recently emerging topic in the field. For instance, absence of viral particle formation does not necessarily mean absence of transcription of viral RNA and translation of viral protein. Even in the case of abortive infection, these

products may have a role in priming the immune system [39].

1.1.1.2. Productive infection – HIV transcription and viral particle production

The most studied scenario of the aftermath of HIV integration is that of productive infection. If an intact proviral DNA sequence encoding replication competent HIV-1 is integrated into the host chromatin, it becomes substrate for the host RNA polymerase II complex.

Long terminal repeats (LTR) on the 5' and 3' termini of the proviral HIV DNA are generated during reverse transcription. A review of the role of LTR sequences in the process of reverse transcription and integration exceeds the scope of this work, and only their importance as an HIV transcriptional regulator will be discussed. The U3 region of the 5' LTR contains the HIV promoter and adjacent regulatory elements involved in recruiting RNA polymerase II, including three tandem SP1 binding sites, a TATA element (together termed the HIV core promoter) and a highly active initiator sequence [40]. In addition, the LTR contains two NF- κ B recognition sites, constituting an activatable enhancer for LTR directed HIV expression. Thus, the HIV LTR is a highly efficient regulator of the initiation of HIV transcription. Most early RNA transcripts of HIV are, however, prematurely terminated and efficient elongation of HIV transcripts requires the viral *trans*-activating factor, Tat [40–42]. Tat activity depends on the presence of the *trans*-activating region (TAR) regulatory element in correct orientation and correct location just downstream of the initiation site [40,42,43]. TAR functions as a transcribed RNA regulatory signal, forming a characteristic stem-loop RNA structure that specifically binds to the Tat protein. In the absence of Tat, the majority of RNA polymerase II complexes stalls near the initiation region, whereas in the presence of Tat, the density of the polymerase downstream is greatly increased. The reason for this is that Tat recruits the cellular positive acting elongation factor b (pTEFb) to the Tat-TAR-pTEFb complex, thereby greatly increasing full-length expression of the HIV genome [40,42,44–46].

HIV primary transcripts undergo complex alternative splicing by cellular factors to produce the full array of mRNA encoding viral proteins. Most HIV-1 strains have 4 (5') splice donor and 8 (3') splice acceptor sites. Splicing of viral RNA is inefficient, producing more than 40 splice variants, and several unspliced transcripts that serve as viral genome or as mRNA for Gag and Pol [23,47]. Incompletely spliced RNA is later translated to Env, Vif, Vpr, Vpu, and completely spliced mRNA transcripts are present for regulatory proteins Tat, Rev and Nef [40,47,48]. Due to retention of intron containing RNA transcripts in the nucleus by cellular splice factors, only completely spliced mRNA may exit the nucleus through host transport mechanisms, and be translated to protein in the cytoplasm, which is why only Tat, Rev and Nef are produced early on after infection. The presence of Tat early on is required for efficient full-length expression of HIV RNA, as discussed above. The role of the viral protein Rev is to facilitate transport of unspliced and incompletely spliced HIV RNA from the nucleus into the cytoplasm [23,47]. Thereby, expression of HIV genes is biphasic, and may be segregated into early (Rev independent, i.e. Tat, Rev) and late (Rev dependent) stages [47].

Gag protein alone, and Gag/Pol polyprotein complexes are the structural proteins required for viral particle assembly. Once the 9 kB full-length mRNA appears in the cytoplasm, a slippery sequence in the transcript ensures a frame shift of the ribosomes in about 5% of the cases into the open reading frame (ORF) of Pol to generate Gag/Pol polyprotein, while 95% of the time Gag is transcribed, thereby setting the optimal Gag to Gag/Pol ratio for particle formation [49,50]. Briefly, Gag and Gag/Pol polyproteins have a role in the following processes: 1.) Trafficking to the plasma membrane sites of viral budding 2.) Capturing and packaging of two copies of full-length viral RNA genome, as well as various viral and cellular proteins that are incorporated into the particle (see above) 3.) Spontaneous polymerization with further Gag and Gag/Pol polyproteins 4.) Autocatalytic cleavage by HIV aspartyl-protease activity in Gag/Pol to form mature viral cores [49,50].

The aspartyl protease activity exhibited by Gag/Pol PR is an essential viral en-

zymatic activity, because cellular aspartyl proteases are ineffective at catalyzing the required cleavages to produce the condensed viral core. This step in the viral life cycle is the target exploited by a class of HIV medication termed protease inhibitors [51].

The viral Env glycoprotein reaches the plasma membrane independent of Gag, and is incorporated into the virion through interactions of Env TM with Gag MA [49].

Viral budding is mediated by the cellular ESCRT (endosomal sorting complexes required for trafficking) pathway, which Gag usurps to facilitate termination of Gag polymerization and catalyze release of the virion [49,50]. Though the classical theory viewed budding of mature infectious virions as the sole way of spreading of HIV, the newly described “virological synapse” may present an alternative possibility. This mode of cell-to-cell transmission may be important within lymphoid tissues, where contacts between T-cells, as well as Dendritic Cell – T-cell interactions may provide a way of direct infection of new target cells without viral particle release [49,52].

1.1.3. Pathophysiology of infection

In this section, the natural course of acute and chronic HIV infection will be discussed, with the exception of full-blown AIDS. An approach with strong focus on pathological T cell dynamics will be applied. Afterwards, alterations of pathophysiology after treatment by cART will be considered.

1.1.3.1. Infection and the acute phase of disease

As outlined above, the most common way of acquiring HIV infection is through heterosexual contact. Male-to-female sexual transmission is estimated to be responsible for the highest number of infections worldwide, and therefore will first be discussed before other routes of infection are referenced briefly. Infection takes place through the genital epithelium or the cervical or uterine mucosa. Free virions, as well as infected

cells from a sexual partner capable of virion release may establish infection. Exact mechanisms for HIV to traverse the epithelium are still debated [53], but HIV may come in direct contact with antigen presenting cells such as Langhans cells (LC) and dendritic cells (DC), macrophages and CD4+ T cells, all of which are abundant in the female genital epithelium and/or the underlying stroma [54]. The role of DC and LC in transporting the virus from mucosal surfaces to CD4+ T cell targets seems important, even though *in vivo* productive HIV infection of these cell types has not been conclusively proven. Rather, the specialized attachment structure present on DC, a C-type lectin termed DC-SIGN binds HIV, and this leads not to the infection of this cell, but to trafficking of the DC and presentation of the virus to CD4+ T cells in lymph nodes, causing infection of the latter [27,53,55]. Invasion by the virus through the vaginal epithelium takes about 30-60 minutes, and it appears to be a focal event, which then leads to viral spreading through the lymphatic system.

In the case of sexual transmission to males, the epithelium and underlying stroma of the foreskin is also rich in CD4+ T cells, LC and DC and may be the most important site of entry for HIV. This would also explain the protective effect of male circumcision [53].

The gastrointestinal tract is a rich source of lymphatic tissue cells that are targets of HIV infection [55,56]. The role of the gastrointestinal tract in initial HIV infection is important as a primary infection site, such as in the case of receptive anal intercourse, and also as a location of initial viral spreading as has been shown by several studies [56,57]. In the case of hematogenous transmission, as may occur in a nosocomial setting or with intravenous drug users, the spleen may be the site where the virus is “filtered” out of the blood stream and comes into contact with lymphatic target cells [53].

Much of the knowledge we have about the T cell dynamics of early HIV infection is based on studies of the simian immunodeficiency virus (SIV) model [55]. After establishment of infection, HIV quickly disseminates locally, infecting primarily CCR5+ CD4+ T cells [55,58]. Homozygosity for a 32 base pair deletion in the *CCR5* gene

abrogates the expression of the viral co-receptor, and confers almost complete protection against infection [13,59]. After two weeks of infection by SIV, increasing numbers of HIV infected CD4+ T cells in distant lymphoid tissue have been reported (lymph node, spleen and mucosal tracts). Lymph node biopsies from small cohorts of acutely HIV infected patients also confirm these findings [55]. The CCR5+ CD4+ phenotype is more frequent in tissue infiltrating T cells, than in peripheral blood or lymph nodes [56], which is why initial spreading of the virus, and establishment of a “reservoir” of HIV predominantly takes place in the gut mucosa-associated lymphoid tissue (see above), estimated to contain 60% of total body T cells at steady state [55].

The abundance of target cells in *de novo* infection causes an initial rise in HIV viremia to proportions that are characteristic only of acute HIV disease. Peak plasma RNA copies in the ten millions per milliliter within three weeks of infection are not uncommon [60]. In the following months, as anti-HIV immune responses develop, viremia declines even without treatment, and reaches within six months to one year of infection a steady state level, termed “set point” [61]. The level of this set point inversely correlates with the time of progression to AIDS in the absence of cART [22].

Interestingly, despite its relevance, the exact mechanism by which HIV infection causes depletion of CD4+ T cells *in vivo* is not fully understood and still debated. HIV and SIV are highly cytopathic viruses, and productively infected cells die *in vitro* by way of HIV induced necrosis [62] or apoptosis [63], while bystander non-infected cells are not affected [62]. *In vivo*, on the other hand, bystander CD4+ T cells that are not themselves infected undergo apoptosis as a result of the general immune activation in the tissue microenvironment, and significantly contribute to the CD4+ T cell depletion that is the hallmark of HIV infection [63–65]. A form of apoptosis that is accompanied by the release of inflammatory molecules is termed “pyroptosis”, and may be the primary form of apoptosis caused by HIV infection [64].

In the pathogenic SIV model, CD4+ T cells in the gut are almost entirely depleted after three weeks of infection [55]. This profound depletion is initially not mirrored by

the loss of CD4+ T cells in the peripheral blood, and presumes infection by a CCR5 tropic virus [66]. Compensatory cell proliferation may keep CD4+ T cell counts in the peripheral blood stable in the acute and post-acute phases of HIV infection [67].

In the acute phase of infection, 50-70% of individuals experience a clinical syndrome around three to six weeks after contracting the virus. Symptoms include fever, skin rash, pharyngitis and myalgia (a “mononucleosis like” disease), their presence correlates with the high level of viremia in early disease, and they subside within weeks as anti HIV immune responses develop and viral loads decrease to steady state. Opportunistic infections may develop at this stage, but are uncommon [22]. Peripheral CD4+ T cell counts are normal to slightly depressed, as discussed above.

1.1.3.2. Chronic HIV infection

HIV infection is considered to have reached its chronic phase, once the level of steady state viremia, the set point has been reached (see above).

The chronic phase of HIV infection is characterized by altogether gradual changes in T cell abundance and viremia. Plasma viral loads are orders of magnitude lower, although constantly rising. In contrast, a significant level of immunological activation persists, and may be an altogether better predictor of disease outcome, than plasma viremia or peripheral blood CD4+ T cell counts [22,55,61].

In the chronic phase, it is unlikely that HIV is directly responsible for CD4+ T cell death, since the degree of productive infection in peripheral blood and tissues is very low, estimated to be 0.01-1%. Furthermore, decay rate of CD8+ T cells and CD4+ T cells in this late phase of the disease appear to be similar, arguing against a direct viral effect [55]. SIV infected sooty mangabeys develop high viral loads, but neither CD4+ T cell depletion, nor progressive disease is observed [55,68]. A heightened state of immune activation is also not observed in this animal model [68–70].

Immune activation as a hallmark of chronic HIV infection may cause T cell depletion through a pathologic chain of events. 1.) Exhaustion of memory T cells by repeated activation. 2.) Compensation by broadly activated naïve T cells constantly converting to memory. These effects lead to an altogether reduced number of resting T cells. 3.) Suppression of lymphopoietic sources, leading to a decreased supply of naïve cells [55,71].

Suppression of the bone marrow – thymus axis in HIV infection is supported by several studies [55,72–74]. X4 strains that emerge in late infection may be especially pathogenic for thymocytes [72,75]. Infected subjects enter the chronic phase with already gravely depleted reserves of CD4+ T cells, especially at mucosal sites (see before), putting an additional strain on homeostatic mechanisms (such as lymphopenia induced production of IL-7 [76]) to keep the bone marrow supplying naïve CD4+ T cells in order to replenish losses.

The question arises: how does HIV cause immune activation in the chronic phase of infection? Viral replication directly causes immune activation by stimulation of T and B cells through viral antigens expressed on infected cells, as well as natural killer (NK) cells through viral products binding to pattern recognition receptors [69,70]. However, as described above, viral replication in chronic infection proceeds at a very low rate, and is probably insufficient to cause immune activation to the extent that is actually observed [69,70]. Even so, markers of immune activation correlate with the levels of plasma viremia [70].

In the previous section, profound depletion of CD4+ T cells in the gut mucosa has been discussed. Low numbers of CD4+ T cells in the gut lymphoid tissue persist throughout chronic infection in untreated HIV disease [55,68–70]. Thus, the epithelial and immunological integrity of the GI tract cannot be maintained, leading to translocation of luminal antigens and various microbial products, such as peptidoglycan, flagellin, bacterial lipopolysaccharide (LPS) into the peripheral circulation. These antigens directly stimulate the immune system to produce tumor necrosis factor (TNF)- α , IL-6,

IL-1 β and type 1 interferons [70] and, thus, contribute to immune activation in humans and in pathogenic SIV models [68,69]. Indeed, LPS levels in peripheral blood have been shown to directly correlate with levels of immune activation in the adaptive and innate immune system [68,70].

Th17 cells are a subset of CD4+ T cells (see later section), characterized by production of a variety of cytokines important in protection against extracellular bacteria and fungi, among them IL-17 being the most characteristic [77]. Th17 cells predominate in the gut, and several studies suggest that this cell type is preferentially depleted even compared to other CD4+ T cells in the gut of HIV infected subjects [78–80]. This particular class of helper T cells has the ability to recruit neutrophils and myeloid cells to effector sites, and is involved in mucosal regeneration, highlighting their importance in preserving the gut mucosal barrier [81], and thus the pathologic significance of their loss in HIV infection [70,81]. The phenotypic characterization of Th17 cells in HIV infection was one of the aims of this work, and will be further discussed later on.

Regulatory T cells (Treg) are another functionally distinct phenotype of CD4+ (also CD8+) T cells. These cells specialize in down-modulating immune responses by other immune cells through immune inhibitory cytokines such as IL-10 and tissue growth factor (TGF)- β , as well as upon cell-cell contact. Treg thus may have a beneficial role in chronic HIV infection, by reducing the level of general immune activation. Injection of IL-2 induces a significant increase in Treg numbers, still clinical trial with recombinant IL-2 in patients on cART could not show a beneficial effect on immune activation [82,83]. On the other hand, Treg may limit anti-HIV immune responses, and thus may have an altogether detrimental role in the context of HIV infection [82,83]. The role of Treg in chronic HIV infection in general, and as a possible reservoir site of HIV in particular, was the major focus of this work, and will be discussed further on.

The chronic phase of HIV infection is symptomatically poor and in untreated patients lasts for a median of ten years [22] before symptomatic immune deficiency (AIDS) develops. Even so, viremia is continuous and progressive during this phase,

with a slow but steady decline in CD4+ T cell counts of around 50 cells/ μ L/year [84]. A rare group of patients, called *long-term nonprogressors* [85] may have symptom free survival times and stable CD4+ T cell counts that are even longer. *Elite controllers* [11,12,86,87] have the characteristic stable CD4+ T cell counts of *long-term nonprogressors* and also maintain plasma viral loads below 50 RNA copies per milliliter (the limit of quantification for most clinical assays). These remarkable individuals are even rarer, representing less than 1% of all HIV patients.

CD4+ T cell counts of less than 200/ μ L carry a risk of opportunistic infections and neoplasms, and the clinical picture of AIDS develops [22]. This scenario will not be discussed.

1.1.3.3. Immune reconstitution and suppression of viremia by combined antiretroviral therapy

Today, combined antiretroviral therapy is recommended by experts for all HIV infected persons, regardless of CD4+ T cell count, based on strong evidence from randomized controlled trials showing a reduction of HIV-related morbidity and mortality independent of the disease stage in which therapy was initiated [8,88,89]. Antiretrovirals are always administered in combination, to prevent development of resistance mutations of HIV. The first treatment regimen should consist of two nucleoside reverse transcriptase inhibitors and a third active component, which is either an integrase inhibitor, a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor [8]. The pharmacologic targets of these different classes of drugs have been briefly described in a previous section on the viral life cycle.

Following initiation of therapy, a rapid tenfold drop of viremia typically ensues within one to two months. This is followed by a more gradual decrease to plasma viral loads below the limit of quantification within six months (50 RNA copies/ml). The rise of peripheral blood CD4+ T cell counts is similarly fast, increasing by 100-150 cells/ μ L in the

first month, and slowly approaching healthy levels afterwards [22]. Early start of cART has beneficial effects on the rate and extent of CD4+ T cell restoration and the level of residual immune activation during therapy, according to many studies [88–93].

Reconstitution of CD4+ T cells in the gut and the mucosal immunological barrier is of pivotal importance in reducing HIV related immunopathology. Whether cART achieves this goal in full is a much debated topic [94–97]. However, evidence exists that early start of therapy may help to restore effective gut immunity [80]. Regeneration of Th17 cell dependent immune responses seem to be of particular importance in this regard [80,94]. Experimental administration of IL-21 in an SIV infection model resulted in expansion of Th17 cells in the gut, and a concomitant reduction of translocation of pathogenic bacterial products and peripheral immune activation, while also prolonging the time to viral rebound after cART interruption in this setting [98].

To conclude, cART is very effective in suppressing viral replication, causing a quick restoration of CD4+ T cell numbers in the peripheral blood, and thereby restoring immunity and preventing the development of AIDS. Furthermore, cART may significantly limit HIV related immunopathology. Still, HIV cannot be cured, because antiretroviral medication cannot achieve a clearance of the latent proviral form of HIV in CD4+ T cells with an immunologically resting phenotype. The side effects of life long therapy by cART are becoming evident as data accumulates regarding patients who have taken this medication for many years [99–101].

1.1.4. The immune response in HIV infection

Several studies have shown, that in around 80% of cases infection of an individual is established by a single virus, termed the founder virus, probably infecting a single focus of mucosal CD4+ T cells [102]. The earliest immune response to HIV is elicited in the innate immune system. Acute phase proteins, such as serum amyloid A appear in the plasma before viral RNA copies reach detectable levels [102]. As viremia in-

creases, so does the level of inflammatory mediators, such as type 1 interferons and TNF α in the blood. NK cells may have an important, albeit unspecific role in the early response against virally infected cells [103]. There is evidence to suggest association between the expression of killer cell immunoglobulin-like receptor (KIR)3D variants and the viral control [103,104].

Within three weeks of infection, viremia in the plasma reaches its peak, and concomitantly, the number of CD4⁺ T cells decreases in the blood, and, to a much larger extent, in the gut lymphoid tissue. Over the course of the following 12-20 weeks, plasma viral loads decline and virus diversification occurs as multiple escape mutants are selected by pressure of the developing adaptive immune responses [102,105,106]. The decline terminates in the establishment of a steady state level of viremia, called the viral set point, which is determined by a balance between virus turnover and the immune response.

Analysis of patients in the very early stage of infection have shown, that most amino acid changes compared to the founder virus in escape mutants that develop early on are selected by CD8⁺ T cells (cytotoxic T lymphocytes, CTL) [107]. At first, immune responses specific for Env and Nef tend to develop, which efficiently clear infected cells and decrease initial viremia [107,108]. However, escape mutants appear very rapidly, caused by the high mutation rate of HIV described in previous sections. Later immune responses by CD8⁺ T cells target epitopes in the more conserved Gag and Pol proteins, and viral escape mutations in these regions come with a so-called “fitness cost” for the virus, meaning a decrease in replicative capacity [102,108]. The viral set point is thus “negotiated” by the efficiency of these immunodominant CD8⁺ T cell responses to target conserved epitopes, and the replicative fitness that the escape mutants are able to retain [102].

CD4⁺ T cells are significantly depleted in HIV infection. Nevertheless, a CD4⁺ T cell response to HIV exists, and fascinatingly, HIV-1 has been demonstrated to preferentially infect HIV-1 specific CD4⁺ T cells [109]. CD4⁺ T cell responses, mainly against

Gag, develop early in infection but are diminished rapidly, along with CD4⁺ T cell decline. CD8⁺ T cell responses decline soon after the founder epitope is eliminated, which may be caused by suboptimal CD4⁺ T cell help and a consequential impaired CD8⁺ memory generation [110]. Early therapy may have the benefit of rescuing CD4⁺ T cell responses against HIV [102].

Regulatory T cells may have a pathophysiological role as a suppressor of anti-HIV immune responses. Treg have been proposed to inhibit HIV-specific CD4⁺ T cell proliferative responses and CD4⁺ and CD8⁺ T cell cytokine production in response to HIV [82,83]. However, correlation could not be detected between Treg frequency and *ex vivo* HIV specific responses in PBMC from untreated HIV controllers or progressors [111]. *In vivo* studies in the SIV model showed a beneficial virologic effect of Treg blockade through administration of an anti-CTLA-4 antibody in rhesus macaques on cART [112]. However, in untreated rhesus macaques with acute HIV infection, CTLA-4 blockade did not increase anti-HIV responses as expected, but rather increased viral replication and infection and loss of CD4⁺ T cells, possibly due to a decreased threshold for CD4⁺ T cell activation [83,112].

B-cell function is severely impacted by the CD4⁺ T cell loss in early HIV infection, because CD4⁺ T cell help is required for germinal center formation and B cell maturation in lymphoid organs [102]. Early antibodies of IgG and IgM class against epitopes of gp120 and gp41 in Env are non-neutralizing, and their effect on plasma viremia is not evident [102]. The first neutralizing antibodies against autologous virus that demonstrably drive viral escape mutations appear late, about 12 weeks after infection [113]. Antibodies that show some degree of neutralization against heterologous virus appear in only 20% of patients, and take years to develop [114].

Broad specificity neutralizing antibodies (bNAbs) against conserved regions of Env are rare, and even if they develop, they usually appear 20-30 months after infection [102,114,115]. Genetic factors, as well as adequate antibody affinity maturation through somatic hypermutation are crucial for the generation of such an immune response. The

latter is a process strongly dependent on CD4+ T cell help, and thus may be hindered in HIV infection [102].

The artificial priming of CD8+ T cell or bNAbs-based immune responses is the focal point of a highly active field of research on a possible protective HIV vaccine. However, no human trial of an HIV vaccine to date, whether bNAbs or T cell based, has been successful in eliciting significant protection against infection [102,115–117].

Host genetics has a considerable influence on anti HIV immunity, and thus the viral set point and the clinical prognosis. Several HLA-B alleles, especially HLA-B*57 and HLA-B*27 are associated with an improved control of the virus, because epitopes recognized by T cells in these individuals focus on conserved regions of the viral Gag protein [118,119]. These alleles are present in significantly higher proportions in cohorts of HIV nonprogressors [118].

1.2. CD4+ T cells and their subdivision

Conventional T lymphocytes are defined by the expression of the $\alpha\beta$ T cell receptor (TCR). T cells mature in the thymus [120]. The maturation involves a complex genome editing process termed VDJ recombination, whereby thymocytes must randomly recombine one of multiple V (variability) D (diversity) and J (joining) DNA segments at the *Tcrb* gene locus and one of multiple V and J segments at the *Tcra* locus to produce a unique TCR [121]. In contrast to B cell receptors, TCR recognize complementary antigen peptides in the context of a major histocompatibility complex (MHC) I expressed on all cells, or MHCII, present on antigen presenting cells (APC) (p:MHCII). Naïve thymocytes are cells that have successfully gone through VDJ recombination, and are expressing a unique TCR, but have not yet been stimulated by a p:MHC for which their TCR has high affinity [121], express both CD4 and CD8, and must undergo positive and negative selection in the thymus before exiting and recirculating through secondary lymphoid organs [122]. Along this process, naïve thymocytes with low affinity bind-

ing to p:MHC I lose expression of CD4 and are positively selected to become CD8⁺ T cells, and those with low affinity to p:MHC II lose CD8 and become CD4⁺ T cells [123]. Thymocytes with high affinity binding to p:MHC are negatively selected, and either die by apoptosis (clonal deletion) or, alternatively, become regulatory T cells (Treg) [122,124]. In the following section, only CD4⁺ T cells will be discussed.

1.2.1. Differentiation and development of CD4⁺ T effector and memory subsets

Naïve T cells (T_n) carrying a TCR with unique antigen complementarity are present in a pool of naïve cells in an individual at a frequency of around 1-7 cells/million, among an estimated total of 3×10^{11} T_n in humans [121,125]. T_n express CCR7 required for trafficking to secondary lymphoid organs, and thus continuously recirculate through the lymphatic system and circulation throughout their lifetime of about 2 years. T_n also express the IL-7 receptor (IL-7R), and are dependent for their survival on IL-7 produced by stromal cells in the lymphoid organs [121,126,127]. Naïve CD4⁺ T cells require activation by their cognate antigen presented on an MHC II molecule expressed on an antigen-presenting cell, usually a dendritic cell (DC) inside a secondary lymphatic tissue (**Figure 1**) [121,126]. Upon high affinity ligation of the TCR by its complementary p:MHC II, TCR molecules aggregate in the so-called supramolecular activating cluster (cSMAC) at the T cell APC contact zone, a process which may be mediated by the interplay of the (APC) CD80/CD86 – CD28 (T cell) co-stimulatory molecules [128] (**Figure 1**). Thus, the immunological synapse between APC and T cell is formed (see previous sections for relevance in HIV transmission, ie. “virological synapse”). This leads to a sequential activation of signal transduction pathways in the naïve CD4⁺ T cell, culminating in the expression of the NFAT and NF- κ B transcription factors, as master regulators of immune cell activation [121,129]. The combination of activating signal 1 (TCR mediated activation) and signal 2 (co-stimulatory signals from APC) dose-dependently cause activation and proliferation of the T_n, resulting in a clonal expansion that increases the frequency of the T_n with a given TCR to 10 000 fold its

original abundance among the naïve repertoire.

To provide a basic overview of CD4⁺ T cell development, the following will present a simplified model of the maturation of CD4⁺ T cells using the example of Th1 differentiation in lymph nodes described in mice. Differences in the case of other phenotypes (ie. Th2, Th17 and Treg cells) will be discussed in the following section.

IL-2 and IL-12 have an important role in early signaling. IL-2 receptor alpha chain (CD25) is upregulated in naïve T cells upon TCR signaling. IL-2 bound receptors activate signaling through Jak1/Jak3 kinases causing the STAT5 transcription factor to activate the expression of the IL-12 receptor β 2 chain (CD25), as well as the T-bet and Blimp-1 transcription factors, meanwhile the cells lose IL-7R (**Figure 1**) [130,131].

Afterwards, the fate of the CD4⁺ T cell may take two different directions [132,133]. According to one model, about half of the cells retain CD25 expression, while the other half loses CD25 expression (possibly through asymmetric cell division) [134,135], and upregulates CXCR5, a chemokine receptor involved in trafficking towards the B cell follicles of lymphoid organs (**Figure 1**) [136]. The CD25⁺CXCR5⁻ cells remain T-bet^{hi}, eventually downregulating CCR7, causing them to exit the lymphoid organ and recirculate to the periphery. These cells become the CCR7⁻ effector T cells (Teff). A minority of the CD25⁺CXCR5⁻ cells will survive the contraction phase of the immune response inside the lymph node, re-express the IL-7R and recirculate to periphery as CCR7⁻ effector memory cells (Tem).

The lack of CD25 expression in the CD25⁻CXCR5⁺ progeny causes a loss of T-bet and Blimp-1 expression. In turn, the transcription factor Bcl6 is upregulated through disinhibition by Blimp-1 [133,136] (**Figure 1**). These cells can again give rise to two different populations depending on the strength of the stimulus they receive through the TCR and the so-called inducible costimulator receptor (ICOS) expressed on their surface. In the B cell areas of the lymph node, the cells may come into contact with B cells displaying the relevant p:MHCII complexes and the ligand for the inducible costimu-

lator receptor (ICOS-L). Cells that thus receive strong signaling through the TCR and ICOS will express programmed death (PD)-1, lose CCR7 and become CCR7-CXCR5+PD-1+ T follicular helper cells (Tfh) [133,136], a T helper cell type that is a major orchestrator of germinal center formation and B cell isotype switching, as well as plasma cell formation. Cells that are not sufficiently stimulated, because of lack of contact with B cells presenting their cognate p:MHCII will become CCR7+CXCR5-PD-1- multipotent central memory cells (Tcm), re-expressing IL-7R and recirculating through the lymphoid organs similar to naïve cells (**Figure 1**) [121,126,132].

Teff cells of the Th1 phenotype lose expression of CCR7, and express chemokine receptors such as CXCR3 and CCR5 [137]. Thus, they recirculate to the site of infection in the periphery, where they come into contact with their complementary p:MHCII presenting macrophages. TCR signaling then results in the production of interferon-gamma (IFN γ), triggering nitric oxide mediated killing of intracellular bacteria in the macrophage [121].

Tem and Tcm cells constitute the immunologic memory, which is required for the secondary response to antigen. Briefly, T memory cells with a given p:MHCII specificity are present at higher frequencies, than Tn (in the range of 1000/cells million) [125]. Furthermore, the threshold for activation of Tem and Tcm cells is lower, and the proliferative responses are faster, because of alterations in the TCR signal transduction pathways [138]. Tem home to peripheral organs, and can mount immediate effector responses at the site of infection. Tcm express CCR7 and recirculate through lymphoid organs, like Tn do, and are able to produce IL-2 and thus expand to a greater degree than Tem cells.

The above presented “asymmetric cell division” model is based on data from mouse models with *L. monocytogenes* and LCMV virus infections. Another model of memory T cell differentiation focuses on the length and quantity of stimulatory signals 1, 2 and 3 on naïve cells to drive the following fates in increasing order of magnitude: increased proliferative capacity (Tcm and Tem), effector function with tissue homing capacity

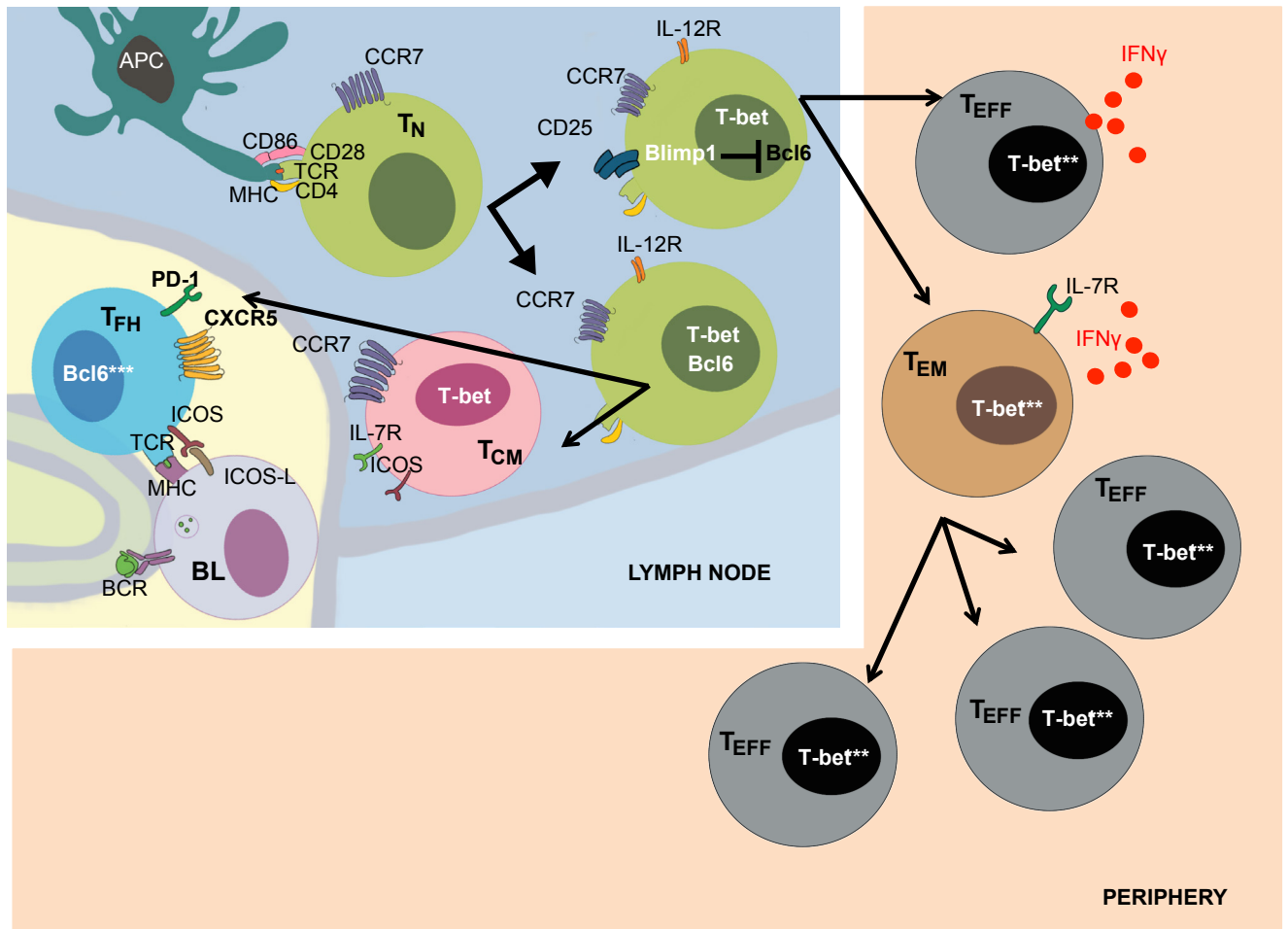


Figure 1. Development of effector and memory subsets from naïve T cells according to a model of asymmetric cell division. See text for details. APC: antigen presenting cell. Tn: naïve T cell. Tcm: central memory T cell. Teff: effector T cell. Tem: effector memory T cell. BL: B lymphocyte. Tfh: T follicular helper cell.

| Subset | Th1 | Th2 | Treg | Th17 |
|----------------------|--------------------------|-------------------------------|------------------------------|------------------------------------|
| Cytokine (inducing) | IFN γ /IL-12 | IL-4 | IL-2 | TGF β , IL-6, IL-21, (IL-23) |
| Transcription factor | T-bet | Gata3 | Foxp3 | Roryt |
| Cytokine produced | IFN γ | IL-4 | IL-10, TGF- β | IL-17A/F |
| Physiological role | Help macrophages and CTL | Parasite immunity B-cell help | Inhibition, immune tolerance | Extracellular bacteria and fungi |

Table 1. Characteristics of CD4+ T cell effector phenotypes. See text for details.

(Teff) or activation induced cell death (“signal-strength” model [139]) .

A linear model of CD4+ T memory cell differentiation (“decreasing potential model” [140,141]) also exists, and is most popular in the HIV field. This will be discussed in more detail under CD4+ T cell reservoirs of HIV. It should be clear, that these three models are not mutually exclusive, and probably elements of all three are to be considered to best describe memory T cell development [138].

CD45 (LCA, leukocyte common antigen) is a molecule expressed on all lymphocytes [142]. CD45 is a protein tyrosine phosphatase, and an obligate positive regulator of signal transduction from the TCR [142]. Alternative splice variants of CD45 are useful to phenotypically differentiate Tn and memory cells. While Tn almost exclusively express the long isoform (CD45RA+CD45RO-), Tcm and Tem are positive for the short isoform (CD45RA-CD45RO+) [143].

1.2.2. Development of functional CD4+ T cell subpopulations

CD4+ naïve T cells may develop different functional phenotypes after TCR stimulation by their complementary p:MHCII (signal 1) and co-stimulation by APCs (signal 2). The cytokine milieu (signal 3) in which Tn cells get stimulated is required for optimal proliferation and differentiation. Signal 3 furthermore determines the functional phenotype of the Teff that Tn develop into [138,144]. Several cytokines have been proposed to have a role in this process, for example IFN α , IFN β , IFN γ , IL-1, IL-4, IL-6, IL-12 and IL-21. Signal 3 cytokines are produced by innate immune cells [138,145], and their nature is determined by the types of pattern recognition receptors that were stimulated by the pathogen or vaccine.

Characteristics of the different functional phenotypes are summarized in **Table 1**.

Th1 cells develop in response to IL-12, as has been discussed above. Furthermore, IL-18 and IFN γ are important stimulators in this phenotype [146]. These cyto-

kines are produced in response to intracellular bacteria and viruses. They activate a signaling cascade in Tn, involving Stat1 and Stat4, leading to production of T-bet, the Th1 master transcription factor [147]. T-bet has many gene targets reinforcing a Th1 fate. The cells express the chemokine receptors CXCR3 and CCR5 [148], responsible for their homing to the gut, skin and joints [149]. IFN γ is the most important cytokine produced by Th1 cells, which is an effector immune cytokine with broad effect, stimulating killing of extracellular bacteria by macrophages, class switching in B cells, and activating CD8 cells and NK cells [146,147,150].

Th2 cells develop in response to IL-4 in the tissue microenvironment, which is also their signature cytokine. Thus, Th2 cells reinforce their phenotype in an autocrine manner. The transcription factor Gata3 drives Th2 development [147]. The cells express CCR3, CCR4 and CCR8 [148] for tissue homing to their target organs. Furthermore, Th2 cells produce IL-13 and IL-15 [146,147,150]. Their broad effects include mobilization of eosinophils, basophils, mast cells and the alternative activation of macrophages, inhibiting the killing of intracellular bacteria. They increase mucosal secretion in the airway and gut epithelium. Thus, Th2 cells are important in defense against helminthes and parasites, but they also drive allergy and asthma [146,147,150].

The recognition of IL-9 producing “Th9 cells” as a separate subset has also been proposed [151], however a transcription factor driving Th9 fate remains to be identified.

In the following, development and function of Th17 cells and Treg will be discussed in more detail, based on their relevance for this work. Tfh cells have been briefly discussed above.

Th17 cells are a CD4⁺ T cell subset with the ability to produce IL-17, a cytokine with broad immunomodulatory function [152,153]. Th17 lineage commitment of naïve cells is initiated by IL-6 and TGF β produced by APC that activate naïve cells. When thus stimulated, Tn start expressing the IL-23 receptor (IL-23R). Further stimulation by IL-23 is

necessary to develop an inflammatory Th17 phenotype [154]. The importance of TGF β is in the suppression of T-bet and GATA-3 (to prevent Th1 and Th2 differentiation), however in higher concentrations it inhibits Th17 differentiation and promotes a Treg fate (see later). IL-6 and IL-23 act through Stat3 to activate the expression of Ror γ t, the master transcriptional regulator of Th17 lineage commitment [154,155].

Negative selection of CD4⁺CD8⁺ thymocytes during naïve T cell development has been discussed above. A mechanism may exist for moderately autoreactive naïve T cells to exit the thymus, and adopt a Th17 phenotype. These cells are termed natural Th17 cells (nTh17), and their peculiarity is an activated/memory - like phenotype despite lack of exposure to cognate exogenous antigen in the periphery [154].

The existence of conventional memory Th17 cells is controversial to some extent. Tem and Tcm retain functional phenotypes of the original effectors they developed from, and consequentially, have a Th1 or Th2 phenotype. On the contrary, Th17 cells readily convert into IFN γ producing cells *in vitro* [156–158] and *in vivo* [159]. Meanwhile, there is no evidence of the reverse occurring [154,159], which makes this phenomenon unique to Th17 cells and calls into question whether Th17 represent a final stage of T cell commitment [154].

On the other hand, there is a considerable body of evidence suggesting that Th17 cells are involved in very long-term recall memory responses against a wide variety of antigens [160]. Furthermore, Th17 show characteristics that are clearly representative of a memory T cell, such as a terminally differentiated phenotype (CD45RO⁺CD45RA⁻CD62L⁻CCR7⁻) with an increased proliferative capacity, and considerable effector potential, such as significant anti-tumor activity in mice [158,159]. Indeed, there is evidence to suggest, that Th17 may be particularly long-lived, and express markers characteristic of stem cells [158,159].

Th17 express chemokine receptors CCR6 and CCR4 [77,161], which determine their homing capacity to their target organs in the mucosal surfaces such as the gut

and lung lymphoid tissue, and to skin [162]. The lack of CXCR3 expression is useful to distinguish Th17 from Th1 cells, that also may express CCR6 [163].

Th17 also carry the C-type lectin CD161, a fascinating receptor molecule for non-MHC ligands in the innate immune system, which identifies cellular subsets capable of IL-17 production across different lymphocyte lineages, such as CD8⁺ mucosa associated invariant T cells (MAIT cells) [164,165], rare T cells expressing the $\gamma\delta$ TCR ($\gamma\delta$ T cells) and the innate lymphocyte NKT cells [166].

Functionally, Th17 are identified by their characteristic production of IL-17 and IL-22. IL-17 denotes a cytokine family (IL-17A, IL-17F and IL-17D) of evolutionarily conserved immune modulatory molecules [167], with a broad range of targets. It promotes recruitment of neutrophils, and is thus an important orchestrator of the immune response against extracellular bacteria and fungi [150]. Th17 are especially important in the defense against *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Candida albicans* [160,163]. Furthermore, IL-17 stimulates the production of defensins, and contributes to germinal center formation of B cells [168]. On the other hand, IL-17 is also an important driver of autoimmunity in systemic lupus erythematosus, rheumatoid arthritis, psoriasis, multiple sclerosis and other disorders [160,169]. IL-22 is an IL-10 related cytokine (see later), with pro- and anti-inflammatory effects. It is important for host defense against *K. pneumoniae*. Th17 cells may produce IL-22, but a subset of CD4⁺ T cells that do not produce IL-17 also produces IL-22. The latter are sometimes termed “Th22” cells [161].

Regulatory T cells are a CD4⁺ T cell subset involved in antigen specific immunologic tolerance [170,171]. Treg have the ability to suppress proliferation and inflammatory responses in other cells. Treg characteristically produce IL-10 and TGF β [172], even though these cytokines are by no means specific to Treg. The developmental biology of Treg represents an exception to the scheme of T_H development previously discussed. Natural Treg (nTreg) develop in the thymus and are positively selected for self p:MHCII engagement [173,174]. nTreg exit the thymus as CD4⁺ T cells with distinctive, mature

phenotype [175]. The first positive signal in nTreg development is TCR engagement by thymic medullary epithelial cells or DCs (signal 1), which is followed by co-stimulatory signal through CD28 (signal 2) [176]. A cytokine milieu is also a prerequisite of effective nTreg commitment (signal 3). IL-2 is the principle cytokine promoting nTreg fate [177], but IL-7 and IL-15 quite possibly contribute [174,178]. Signaling cascades through Jak3 and STAT5 are initiated, finally inducing expression of FOXP3 [177], the master transcriptional regulator of the Treg phenotype. IL-2 is continually required for the survival of Treg in the periphery [130].

There is considerable evidence to support the model, in which Treg can also be generated in the periphery, in a thymus independent manner [131,174,179,180]. TGF β can induce Foxp3 as well as Ror γ t expression in naïve T cells *in vitro*. Whether the Tn then develop a Treg or a Th17 phenotype is dependent on the co-administration of IL-2 or IL-6, respectively [173]. There is evidence to support that TGF β regulates iTreg generation *in vivo* [181]. iTreg are generated from naïve T cells stimulated by contact with non-activated DCs that present low doses of p:MHCII over prolonged periods of time (such as in the case of chronic viral infections, or diet based allergens) [182,183]. The gut mucosal lymphoid tissue might provide especially favorable conditions for iTreg formation [180].

Foxp3⁺ versus Foxp3⁻ CD4⁺ T cells in mice display almost completely different TCR repertoires [184], which is suggestive of the different thymic origin (positive versus negative selection) of Treg and non-Treg CD4⁺ T cells. At the same time, these findings [184] suggest that most Treg develop as natural nTreg. The question of the relative contributions of these two subsets is a challenging one, as there are no cell surface markers to distinguish iTreg from nTreg. The transcription factor Helios has been proposed to be expressed by nTreg, but not iTreg in mice [179,185,186]. Significant differences may also exist between mice and humans. TCR based clonal comparisons between Treg and non-Treg similar to those mentioned above have been performed in memory cells of humans as well, and suggest that a more significant proportion of

human Treg may be non-thymus derived [187], but further studies in this area are definitely warranted.

The phenotypic identification of Treg in mice is straightforward, and Treg can be identified with reasonable specificity based on surface expression of the IL-2 receptor alpha chain, CD25 [171,175,177]. Alternatively, Treg are defined as cells expressing intracellular Foxp3 [188]. The question of Treg identification in humans is somewhat more complicated, as CD25 in humans is also expressed on activated non-Treg CD4+ T cells [189]. Thus, it was proposed, that a low expression of the IL-7 receptor (CD127) should be included as a criteria for Treg definition based on cell surface markers [189,190]. However, as discussed before, the IL-7 receptor is downregulated early on in all activated T cells, and thus, the CD127^{lo} phenotype is unlikely to be Treg specific. The surface marker combination CD4+CD25+CD127^{lo} identifies a cell population that is 85-90% FOXP3+ [173].

The exact mechanisms of Treg mediated suppression remain elusive to our understanding. Treg can efficiently suppress activation and proliferation of non-Treg T cells under experimental conditions. Interestingly, neutralization of IL-10 and TGF- β does not abrogate Treg mediated suppression, but separation by a semi-permeable membrane does [191]. It follows, that cell-cell contact dependent mechanisms must be necessary for Treg function *in vitro*.

Treg express several cell surface molecules, which may be involved in their inhibitory function. These involve cytotoxic T lymphocyte associated protein 4 (CTLA-4), and lymphocyte activation gene 3 (LAG-3), both of which have been proposed to play a role in mechanisms in which Treg inhibit the co-stimulatory function of dendritic cells, and thus activation of Tn [172,173]. Furthermore, murine Treg express CD39 and CD73 [192], two ectoenzymes that act in concert to convert adenosine-triphosphate (ATP), a stimulatory molecule for DC, into adenosine, an inhibitory molecule for DC and T cells [174]. Human Treg also express CD39, but not CD73 [192]. Finally, Treg may also kill effector cells, by releasing perforin and granzyme A [172].

The importance of IL-10 *in vivo* is supported by mouse experiments, where Treg lacking IL-10 were unable to protect from autoimmune colitis [172,173]. There is evidence, that TGF β may be present on the surface of Treg, and thus has been proposed to have a role in cell contact dependent suppression [172]. The *in vivo* importance of TGF β in this regard, however, is unclear.

1.2.3. CD4+ T cell plasticity

The original paradigm of the Th1/Th2 dichotomy used to explain the nature of CD4+ T effector responses was called into question after the description of Treg cells and Th17 cells [150].

The view of single transcription factors guiding single lineage commitment has to be altered to explain a mounting body of evidence showing that CD4+ T cells may, in fact, express multiple “master” transcriptional regulators at the same time [193,194], and flexibly change their phenotype in different pathophysiological settings.

For example, committed GATA3+ Th2 cells can adapt a Th1 like phenotype, with expression of T-bet and IFN γ [195]. Tfh cells can be differentiated into Th1, Th2 or Th17 cells *in vitro*, and the reverse is also possible [193]. *In vivo*, FOXP3+ cells may differentiate into Tfh cells in Peyer patches of the gut [196].

In the following, Th17 and Treg plasticity will be discussed in further detail.

Th17 cells are probably the most well recognized as a subset with inherent plasticity, since their ability to adopt an IFN γ secreting (Th1-like) phenotype has been confirmed by a number of *in vitro* [156,163,197,198] and *in vivo* [159] studies, particularly in inflammatory conditions and at sites of inflammation [199,200]. Adopting the Th1 phenotype causes Th17 cells to secrete IFN γ , accompanied by a loss of IL-17 production and continued expression of CD161 [156,200], while the cells may lose [197] or maintain [156] CCR6 expression. *In vitro* experiments with sequential exposure of Tn

to Th17 (IL-6 and TGF- β), followed by Treg (IL-2) polarizing conditions have demonstrated that Th17 can be *trans*-differentiated into IL-17+ cells that express FOXP3 and CD39, and show significant suppressive capacity [201,202]. The *in vivo* relevance of these suppressor Th17 cells is not yet clear.

Treg cells are also known to adopt effector T cell profiles. This may happen *in vitro*, when Treg are cultured under conditions that promote differentiation into Th17 [203], or *in vivo* in adoptive transfer experiments into lymphopenic host mice [204], where Treg have been shown to differentiate into cytokine producing effectors of Th1, Th2 as well as Th17 lineages [204]. These dogma-challenging studies evoked some controversy, since Treg are positively selected in the thymus based on autoreactive TCR repertoire, and their reprogramming to become effector cells would result in serious autoimmune pathology [205]. Consequently, these findings have been challenged on methodological grounds, showing that the Treg population is phenotypically stable [206], as well as by findings showing completely different TCR sequences in Foxp3+ vs. Foxp3- cells [207]. To reconcile these controversial studies, it has been proposed that a multilevel process, including epigenetic modification of the *Foxp3* locus, controls Treg differentiation and a subset but not all of Treg may remain reversibly committed to the Treg fate [205].

1.3. CD4+ T cell reservoirs of HIV in chronic infection

The advent of cART has rendered HIV a chronic, manageable condition in countries with wide spread access to the life-saving medication [3]. HIV remains, however, an incurable disease. Patients receiving cART may have no clinical laboratory evidence of viral replication for many years. Still, when medication is withdrawn, viremia rebounds within weeks. Memory CD4+ T cells with a resting phenotype have been proposed to be a major reservoir of virologically latent HIV [208]. Indeed, proviral HIV DNA can be detected by polymerase chain reaction (PCR) in these cells in individuals

on suppressive cART [208,209]. Furthermore, latent proviral HIV could successfully be reactivated *in vitro*, and replication competent virus cultured from resting memory CD4⁺ T cells, thereby confirming their role as a latent reservoir of HIV [210].

The following section will briefly discuss the possible molecular mechanisms behind HIV latency, the biological phenomenon responsible for the persistence of HIV reservoirs despite therapy. Afterwards, the pathophysiology of HIV reservoirs will be discussed, followed by the formulation of the hypothesis for this work, namely, that the CD4⁺ T cell population is heterogeneous with regards to proviral HIV burden.

1.3.1. HIV latency and the HIV reservoir

According to one broadly accepted model, HIV preferentially infects activated CD4⁺ T cells. Higher infectibility of activated cells has been demonstrated by *in vitro* experiments [211–213]. Furthermore, phenotypic characteristics of activated T cells also provide theoretical support for this view [214], such as higher CCR5 expression [58], and the presence of the restriction factor SAMHD1 in resting cells [215]. According to this model, activated naïve T cells in the host are infected by HIV, possibly at a “sweet spot” for infection during a phase of transition from an activated to a resting memory state. This favors reverse transcription and integration, but not transcription of the HIV genome [214,216]. Afterwards, the cells adopt an immunologically resting phenotype with low level of general gene expression, and several other characteristics that restrict HIV transcription, and thus latent HIV infection is developed [214,216,217]. The generation of resting Tn during thymopoiesis also represents a reversion from an activated to a resting state, and infection of CD4⁺CD8⁺ thymic progenitors with subsequent development of latency has been shown in HIV – infected humanized mice [218].

Several studies have proposed an alternative way of establishing latent infection, according to which HIV may directly infect resting naïve and memory cells. This model is based on successful experimental infection of resting CD4⁺ T cells. Cytoskeleton

rearrangement in latent cells is important to become permissive to infection [219], and the successful *in vitro* infection of resting CD4+ T cells by co-administering CCL19 and CCL21 (ligands of CCR7) to induce these changes seems to support this notion [213,219–221]. The significance of the cytokine milieu is also suggested by studies showing that resting CD4+ T cells from lymphatic tissue can be infected *in vivo* in the context of other lymphoid cells of the tissue [212], but not *ex vivo* in purified form [222]. Others have succeeded in demonstrating HIV integration after *in vitro* infection of resting CD4+ T cells [211], showing no effect of CCL19 on infection rates, and confirming a state of latent infection with the possibility of HIV reactivation in a fraction of these cells [223].

In light of data supporting both of the above models, it is probable that infection of activated T cells with subsequent reversion to a resting state, as well as direct infection of resting CD4+ T cells contribute to the establishment of latent HIV infection *in vivo*.

As discussed before, HIV preferentially integrates into the introns of actively transcribed genes. Multiple mechanisms have been proposed that might limit HIV transcription, and thus maintain a state of latency.

Transcriptional interference denotes a state, where the spatial proximity of the integrated HIV provirus to an actively transcribed gene causes an inhibition of transcription from the HIV promoter [214,217]. Mechanisms, such as promoter occlusion (transcription of an active gene upstream of HIV causes a “readthrough” by host RNA Polymerase II, displacing Sp1 from the HIV promoter), convergent transcription (HIV integrates in an opposite orientation to a host gene, causing collision of the RNA Polymerases from host and viral promoters) may be responsible [217].

The transcription factors NF- κ B, NFAT and SP1 are key initiators of HIV transcription, as described above. It may be very relevant with regards to HIV latency, that these factors are sequestered in the cytoplasm in resting CD4+ T cells [224], and are thereby unable to promote HIV transcription [217]. Compounds such as prostratin act through

the protein kinase C pathway to activate NF- κ B signaling, and have been shown to reverse HIV latency [225].

The viral *trans*-activator protein Tat recruits the cellular transcriptional elongation factor pTEFb for successful transcriptional elongation during HIV transcription, an essential mechanism for HIV replication. pTEFb is inactive in resting CD4⁺ T cells, because its components are bound to the inhibitory molecule HEXIM1 [214,217,226,227], and blocking of HEXIM1 has been shown to cause HIV reactivation from resting CD4⁺ T cells [228].

Epigenetic regulation is likely to play a significant role in HIV latency, and is not well understood to date. Epigenetic regulation through histone modifications on nucleosomes - the structural unit of chromatin, consisting of 147 base pairs “coiled” around a complex of histone proteins – may, however, allow for a more universal control of regulation of gene expression [217,227]. Heterochromatinization (tight binding of proviral DNA to nucleosomes) at the site of HIV integration may present a way of silencing of HIV gene expression, and has been demonstrated in a cell line model of latency [229], as well as in primary CD4⁺ T cells [226]. On the other hand, as discussed above, HIV proviral DNA is predominantly found in the introns of actively transcribed genes [33], which argues against heterochromatinization as a major mechanism of HIV latency. Two nucleosomes consistently form at the 5'LTR of the HIV proviral DNA [230], even if the provirus is integrated into euchromatin (a relaxed configuration of DNA-nucleosome complexes), and the maintenance of one of these, nuc-1, is dependent on the state of histone acetylation [217,227].

An experimental therapy using histone deacetylase (HDAC) inhibitors for reactivation of latent HIV is effective in reversing HIV latency through disruption of nuc-1, and subsequent recruitment RNA polymerase II to the HIV promoter [217,227]. After promising initial results [231], treatment of HIV infected patients on intensified cART with valproic acid, an HDAC inhibitor, did not appreciably decrease the size of the latent reservoir [232,233].

Methylation of the DNA at CpG dinucleotides by DNA methyltransferases is another epigenetic mechanism, by which expression of genes may be repressed. CpG methylation occurs in the 5'LTR of the provirus, where it most likely inhibits binding of transcription factors NF- κ B and SP1 [217,227]. Synergistic activation of HIV transcription could be achieved in latently HIV infected cell lines by co-administration of aza-CdR, an inhibitor of DNA methylation, and prostratin, an activator of NF- κ B [234]. On the other hand, when compared to cell lines, CpG methylation was detected only in a very low level in resting CD4+ T cells of HIV infected individuals on cART [235], suggesting an overall smaller significance of DNA methylation as a main mechanism for HIV latency *in vivo*, than previously thought.

1.3.2. Identified anatomical and cellular reservoirs of HIV

As discussed above, HIV may persist in its latent form predominantly in resting memory CD4+ T cells. Even though non-CD4+ cells may also contain latent HIV provirus, the reservoir in the pool of memory CD4+ T cells, with a half-life of 44 months [236], seems to be sufficient to ensure life-long persistence of HIV despite cART. An HIV reservoir has been described in various non-T cells of the central nervous system, and at other sites in macrophages [209,237] and recently in $\gamma\delta$ T cells [238].

Peripheral blood CD4+ T cells are easily accessible for research purposes, and have provided valuable information to our understanding of the HIV reservoir. Still, it should be considered, that less than 2% of total body CD4+ T cells are found in the circulating blood [55,239], while their overwhelming majority is located in lymphoid organs, such as lymph nodes and the gut mucosal lymphoid tissue. Furthermore, some tissues may represent sanctuary sites, based on suboptimal penetration of antiretroviral drugs (for example in lymphatic tissue) [240], or the existence of an immunologic barrier (such as in the central nervous system, CNS) [241].

While the clearance of virus in lymph nodes was only found to be slightly longer

in lymph nodes, than in blood, a higher viral reservoir in lymph nodes and spleen has been shown in the SIV model [239,242]. In humans, the relative HIV burden in CD4+ T cells may not be higher in lymph nodes [209], but considering that a much larger proportion of total CD4+ T cells resides in lymphoid tissue, than in blood, the absolute contribution of the lymph-node-associated CD4+ T cell reservoir may still be more significant. Furthermore, lymph nodes contain most of Tfh cells, a CD4+ T cell subset proposed to be important as an HIV reservoir [243,244]. Tfh cells are located at the center of germinal centers in lymph nodes, areas where cART drugs may reach lower concentrations [239,240], and which may be protected from access by HIV specific cytotoxic CD8+ responses [245].

Initial spreading of HIV takes place in the gut, responsible for establishment of the reservoir [55]. Indeed, multiple studies have confirmed a CD4+ T cell reservoir of HIV infected cells in the gut [94,96,97,239,246,247], with some studies showing a range of tenfold higher HIV burden as in blood [96,246]. Evidence exists to support the role of a non-CD4+ gut reservoir as well [247].

The central nervous system represents another important reservoir and sanctuary site for HIV persistence [248], with pathophysiological consequences (AIDS dementia complex [249]). The *in vitro* infection of many different cell types in the CNS have been demonstrated, including astrocytes, fetal neural cells, microglia and capillary endothelial cells [239]. Furthermore, transient productive infection, followed by a reversible latent infection of astrocytes and astroglial cells has been described [250].

As discussed before, CD4+ T cell maturational stages (Tn, Teff, Tem, Tcm) as well as functional phenotypes (Th1, Th2, Th17, Treg), are initiated by distinctive transcriptional programs. Given the heterogeneity of the surface receptor expression profile, homing potential and the transcriptional landscape in these various CD4+ T cell subpopulations, a difference in permissiveness to HIV infection, preponderance for latent infection as well as reactivation potential of latent provirus has been hypothesized in several studies [251–255].

Models for the development of CD4⁺ T effector and memory subsets from T_n have been presented in a previous section. In recent years, a slightly different model, proposing a sequential development of CD4⁺ T memory subsets has emerged, which supposes a certain linearity, whereby subsets evolve from each other in a unidirectional fashion (broadly: T_n→T_{cm}→T_{em}→terminally differentiated effector [T_{td}/T_{te}]). This developmental model has enjoyed increasing popularity since the description of “stem-cell like” memory cells (T_{scm}) [256], and is further supported by observations of decreasing proliferative capacity [255,256], corresponding to decreasing telomere length [141,257], and an increasing number of differentially expressed transcripts compared to T_n [256] along the linear development proposed. Furthermore, an intermediate phenotype “between” T_{cm} (CCR7⁺CD27⁺) and T_{em} (CCR7⁻CD27⁻) has also been described (CCR7⁻CD27⁺) [257–259], and termed transitional memory cells (T_{tm}) [259]. Several experts now propose differentiating between a total of six T cell memory subsets, according to the linear developmental model: T_n→T_{scm}→T_{cm}→T_{tm}→T_{em}→T_{td}(T_{te}) [141,255,259]. In the field of HIV reservoirs, this model is particularly well accepted [251,253,255].

Theoretically, proviral DNA that is capable of reactivation and renewed production of virus guarantees the persistence of infection for the lifetime of the cell. Thus, long-lived cellular CD4⁺ T cell subsets may be of particular interest as reservoir sites of HIV.

In 2009, Chomont and colleagues provided evidence for the first time, that the long-lived subsets T_{cm} and T_{tm} were the major sites of HIV persistence in patients on long term antiretroviral therapy [251]. In patients with CD4⁺ T cell counts at near-healthy levels, integrated HIV proviral DNA was mainly found in the T_{cm} subset. In patients with lower CD4⁺ T cell counts, the reservoir was shifted towards the T_{tm}, and, to a lesser extent, T_{em} subsets. The proviral DNA burden in T_n and T_{td} was much smaller. Furthermore, the authors showed data suggesting that T_{tm} cells carrying proviral DNA were differentiated from infected T_{cm}, based on phylogenetic relationship between viral sequences in these two populations that was present in patients with lower CD4⁺ T

cell counts and actively proliferating CD4⁺ T cells, but not in patients with higher CD4⁺ T cell counts, where CD4⁺ T cells tended to have a resting phenotype [251]. The work proposed a mechanism of IL-7 driven homeostatic proliferation characteristic to T_{cm}, to maintain a genetically stable reservoir in patients when viral replication is fully suppressed by cART. The significance of T_{cm} and T_{tm} as a major reservoir has since been confirmed by others [260–262].

The recognition of the importance of a long half-life, as well as homeostatic proliferative capacity generated interest in the newly described T_{scm} subset as a possible reservoir site of HIV. Two research groups have reported that T_{scm} cells may represent a particularly long lasting site for HIV persistence [253,263]. The overall low abundance of T_{scm} cells, and the fact that their phenotypic identification is different from study to study represents some controversies [253,256,263].

Th17 cells have been known to be readily infectible by HIV for some time [252,264,265]. Even though these cells are depleted in the gut mucosa in early infection (see above), some studies show restoration of the subset during long-term cART [94,266,267], or their protection by early therapy [80]. Stem-cell like properties and a long half-life were described for Th17 cells [158–160], which makes their possible role as an HIV reservoir an interesting question to investigate [253,255]. Still, very limited information exists on the proviral DNA burden of Th17 cells [78,252], while no data on replication competent viral reservoirs in this subset are available. The transcription factor PPAR γ has been proposed as a restriction factor for HIV transcription in Th1-17 [268] and Th17 [252] cells, providing a molecular clue to hypothesize that Th17 cells are a possible latent reservoir of HIV in patients on cART.

Treg cells are readily infectible by HIV *in vitro* [269,270] and a possible role of Treg as a reservoir of HIV-1 in chronic infection has been proposed [254,271]. In particular, proviral DNA loads in sorted regulatory T cells identified by the CD25⁺CD127^{lo} phenotype in HIV patients on (cART) have been reported higher than that of non-Treg CD4⁺ T cells [254,271]. Furthermore, the presence of replication competent virus in Treg was

qualitatively demonstrated. However, the fraction of Treg cells harboring replication competent virus has not been quantified to date. In contrast, others did not find a significant difference in HIV DNA copy numbers between Treg and non-Treg CD4+ T cells in a similar cohort [272]. In untreated patients with viremia, Treg proviral DNA burdens were reported to be higher than in naïve cells, but not compared to memory CD4+ T cells [273]. Finally, overexpression of Foxp3 was experimentally shown to restrict HIV transcription from the LTR in the cell [274,275], providing *in vitro* rationale to hypothesize Treg as a preferred reservoir.

1.3.3. Hypothesis: Heterogeneity of CD4+ T cell subsets with regards to proviral burden

In light of previously discussed findings, the CD4+ T cell population should not be regarded as a homogenous group of cells. Rather, it is made up of various subpopulations representing subsets of different functional profile, and different stages of development. Conversely, these subpopulations are all characterized by a markedly different transcriptional milieu and epigenetic landscape.

Many cellular pathways involved in the regulation of T cell function and activation have been suggested to have a role in the regulation of HIV latency. Thus, it is fair to assume, that HIV latency is differentially regulated in CD4+ T helper subsets and memory subpopulations with different transcriptional profiles.

Treg and Th17 cells are likely candidates to be preferential reservoir sites of HIV in patients on cART. Since these subpopulations represent a minority of all CD4+ T cells, their pathological importance as reservoir sites of HIV can only be established, if it can be demonstrated that the proviral HIV burden in these cells is significantly higher, than in Tcm and Tem, the major CD4+ T cell subpopulations harboring latent HIV.

The aims of this work were as follows: 1.) Perform a phenotypic characterization of the Th17 and Treg population in a cohort of untreated HIV patients with ongoing virus replication, as well as patients on combined antiretroviral therapy. 2.) Establish sensitive assays for quantification of proviral HIV DNA and replication competent virus in small CD4+ T cell subsets from clinical samples. 3.) Live-sort multiple phenotypically pure CD4+ T cell subsets, such as Treg, Th17, Tcm and Tem in a cohort of HIV patients with long-term complete suppression of viremia by cART, to assess and compare proviral HIV DNA burden and the replication competent reservoir in these subpopulations.

2. Materials and Methods

2.1. Processing of clinical blood samples

Healthy donors and HIV infected patients were recruited at the University Medical Center Hamburg-Eppendorf (Hamburg, Germany). Written informed consent was obtained from all study participants. Procedures were approved by the local Institutional Review Board of the Ärztekammer Hamburg, Germany. Clinical data including patient history, peripheral blood CD4⁺ T cell counts, plasma viral loads and treatment regimens were extracted from the clinical database for use in the studies. COBAS AMPLICOR assays (Roche Molecular Systems) were used to determine HIV viral loads, with a limit of quantification of 20 RNA copies/ml plasma.

40 ml full blood was drawn into Vacutainer Cell Preparation Tubes (BD Biosciences), and PBMC was isolated according to the manufacturer's instructions, by centrifugation (20 minutes, 2000 x g).

2.2. Freezing and thawing of PBMC

PBMC were processed fresh for experiments involving fluorescence-activated cell sorting (FACS). In other cases, PBMC were resuspended in freezing medium containing 25% fetal calf serum, 65% Roswell Park Memorial Institute 1640 Medium (RPMI), 10% dimethyl-sulfoxide, and cryopreserved as $0.5-1 \times 10^7$ cells/1 ml aliquots. Aliquots were stored in liquid nitrogen until processing.

To thaw, cryopreserved PBMC samples were placed in a 37°C water bath for one minute and then resuspended in 50 ml of pre-warmed (37 °C) RPMI by gentle pipetting. PBMC were pelleted by centrifugation, diluted in 1xPBS and counted, then washed once with 1xPBS.

2.3. Immune-phenotypic analysis

2.3.1. Cell surface staining of immunologic markers

Of each sample, 1×10^6 cells were used for surface staining, and in parallel at least 2×10^6 were used for ICS. All cells were stained with the Zombie Yellow™ Live/Dead discrimination dye according to the manufacturer's instructions (BioLegend). Subsequently, samples were stained with a panel of fluorochrome-conjugated antibodies against various surface markers: anti-CD161 (FITC, clone HP-3G10, 1.25:100), anti-CCR5 (PerCP/Cy5.5, clone HEK1/85/a, 1.5:100), anti-CD25 (PE/Dazzle™594, clone M-A251, 1:100), anti-CCR4 (PE/Cy7, clone L291H4, 1:100), anti-HLA-DR (Alexa Fluor® 700, clone L243, 5:1000), anti-CD4 (APC/Cy7, clone SK-3, 1:100), anti-CCR6 (Brilliant Violet 421™, clone G034E3, 2:100), anti-CXCR3 (Brilliant Violet 510™, clone G025H7, 3:100), anti-CD127 (Brilliant Violet 605™, clone A019D5, 1.25:100), anti-CCR7 (Brilliant Violet 650™, clone G043H7, 1.8:100), anti-CD3 (Brilliant Violet 785™, clone OKT3, 1:100) (all BioLegend) and anti-CD45RO (BUV395, clone UCHL1, 1.25:100, BD Biosciences). Samples were incubated for a total of 30 minutes at room temperature in the dark, then washed once with 1xPBS containing 2% FCS. Samples were fixed with 0.5% paraformaldehyde and stored overnight at 4°C. The following morning, samples were washed once more with 1xPBS and were then resuspended in PBS for analysis by flow cytometry.

2.3.2. Intracellular cytokine and transcription factor staining

PBMC were resuspended in X-vivo 15 medium (Lonza) supplemented with Fetal Calf Serum (10%), L-Glutamine (200 mM) and Penicillin/Streptomycin (5000 U/ml/5000 µg/ml). Samples were plated overnight at 37°C and 5% CO₂. Next morning, cells were stimulated with phorbol myristate acetate (PMA) and ionomycin (both Sigma-Aldrich)

at a concentration of 50 ng/ml and 1 µg/ml respectively. A portion of each sample was handled separately and was left without stimulus (unstimulated control). A Golgi-block cocktail of brefeldin-A (1 mg/ml, Sigma-Aldrich) and monensin (2 mM, BioLegend) was added, then samples were incubated at 37°C and 5% CO₂ for six hours. Subsequently, the medium was washed off and cells were resuspended in 1xPBS.

Live/Dead dye (Zombie Yellow™) and surface antibody staining was performed as described above, with the following antibodies: anti-CCR7, anti-CD8, anti-CD3, anti-CD45RO (clones, fluorochromes and concentrations as described above). Afterwards, cells were fixed and permeabilized with the FOXP3 Fixation/Permeabilization Buffer Set (eBioscience) according to the manufacturer's instructions. Subsequently, samples were stained with the fluorochrome-labeled antibodies anti-IFNγ (PE/Cy7, clone 4S.B3, 3:100), anti-CD4 (APC/Cy7, clone SK3, 1:100), anti-IL-17A (Brilliant Violet 421™, clone BL168, 2.5:100) (all BioLegend) and incubated for 30 minutes at 4°C in the dark. Samples were washed once, then resuspended in 1xPBS.

2.3.3. Flow cytometry analysis

All samples were analyzed on a BD LSR Fortessa flow cytometer running FACS Diva version 6 (BD Biosciences, Heidelberg, Germany) on an IBM based PC workstation. The panel was compensated using single-stained Comp Beads (Anti-Mouse Ig, κ/Negative Control Compensation Particles Set, BD Biosciences, Heidelberg, Germany). For Live/Dead compensation, Comp Beads stained with anti-CD45RA (Brilliant Violet 570™, clone HI100, 0.5:100, BioLegend) were used.

2.4. Fluorescence-activated cell sorting

Peripheral blood mononuclear cells (PBMC) were obtained from blood donations of around 50 ml by Ficoll gradient centrifugation using Vacutainer Cell Preparation

Tubes (BD Biosciences). Monocyte depletion of the cells was carried out as previously described [279]. Magnetic negative enrichment of CD4⁺ T cells was performed using biotin labeled antibodies against CD8 (clone: RPA-T8, 5:100), CD14 (clone: HCD14), CD19 (clone: HIB19), CD16 (clone: 3G8), CD235 (clone: HIR2). All antibodies were added in a 5:100 dilution, and were purchased from BioLegend. Cells were incubated with the antibodies for 25 minutes at room temperature, and then washed once. Afterwards, Streptavidin Dynabeads (Life Sciences) were added in a concentration based on cell numbers as recommended by the manufacturer, and the cells were incubated for 25 minutes, before magnetic enrichment was performed using the “Big Easy” EasySep™ Magnet (STEMCELL Technologies). The enriched fraction was stained with Live/Dead Near-Infrared Dead Cell Stain Kit (Life Sciences, 1:100 dilution) and a panel of antibodies against CD45RO (FITC, clone: UCHL1, 5:100), CD25 (PE/Dazzle™594, clone M-A251, 5:100), CD127 (PE/Cy5, clone: A019D5, 6.5:100), CCR7 (PE/Cy7, clone: G043H7, 1:10), CD3 (Alexa Fluor 700, clone: OKT3, 5:100) (all BioLegend) and TCRγδ (APC, clone 11F2, 1:10, Miltenyi Biotech). The same clones that were applied for magnetic enrichment were used to stain the following (dump channel, APC/Cy7): CD8, CD19, CD16 (5:100), CD14 (6.5:100). Cells were incubated for a total of 30 minutes with the staining reagents, then washed once and resuspended at 10⁷/ml in RPMI containing 10% FCS for the sort.

The cells were sorted by FACS for live CD3⁺CD8⁻CD14⁻CD16⁻CD19⁻TCRγδ⁻ (CD4⁺) CD25⁺CD127^{lo} (Treg), and non-Treg CD45RO⁺CCR7⁺ (Tcm) and CD45RO⁺CCR7⁻ (Tem) on a FACS Aria Fusion (BD Biosciences). The gating strategy is shown in **Figure 2**. TCRγδ antibody was acquired from Miltenyi Biotech, all other antibodies were from BioLegend. Post-sort purities of more than 95% were achieved.

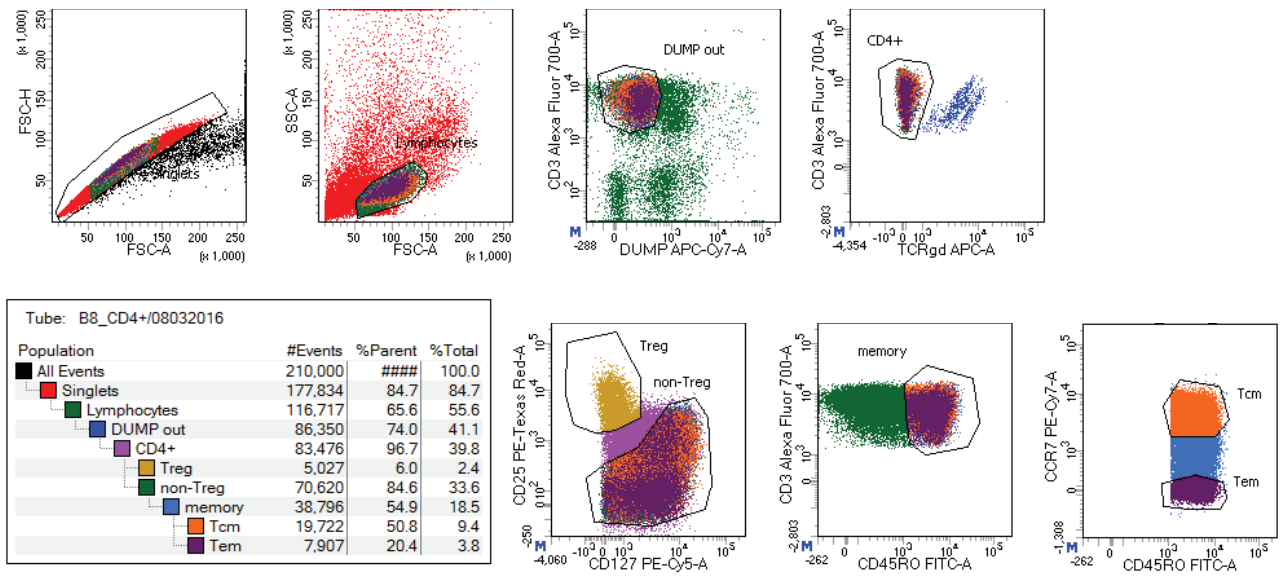


Figure 2. Gating strategy for FACS sort of Treg, Tcm and Tem. Backgating on live CD14-CD16-CD19-CD8-TCR $\gamma\delta$ -CD3+ lymphocytes. Tregs identified as CD25+CD127^{lo}. Non-Treg memory cells (CD45RO⁺) are sorted as Tcm (CCR7⁺) and Tem (CCR7⁻).

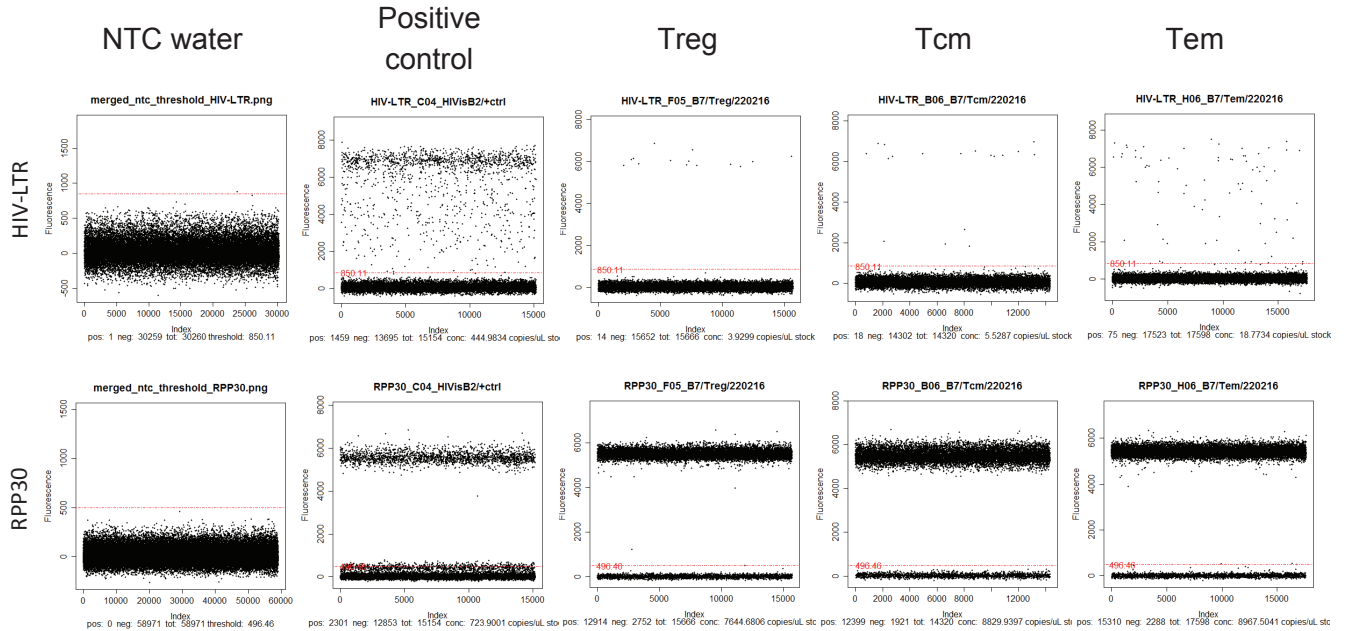


Figure 3. Droplet Digital PCR threshold determination for HIV-LTR copy number quantification in CD4+ subsets. ddPCR dot-plots from a representative experiment, measuring HIV-LTR and RPP30 copy numbers in duplex, showing automated thresholding by the ddPCRquant tool based on fluorescence data of the no template control (NTC) well droplets.

2.5. Quantitative real-time PCR based quantification of HIV-1 proviral DNA

PBMC samples were thawed as described above. DNA was purified using the DNA Micro Kit (Qiagen) according to the manufacturer's instructions. DNA concentrations were determined using a Nanodrop 1000 spectrophotometer (Thermo Scientific).

Quantitative real time PCR was performed using the Generic HIV DNA Cell kit (Bio-centric), according to the manufacturer's instructions with minor modifications. Briefly, patient derived DNA samples were assayed in duplicates of maximum 1 µg pro reaction, as well as in a 1:10 dilution using primers and probes binding in a conserved region of the 5'LTR as described [277]. A standard curve was generated using serial dilutions of HIV-1 viral DNA prepared from the 8E5 cell line, diluted in human background DNA in \log_{10} decrements (reagents provided by the kit manufacturer). Platinum TAQ Supermix was provided by the kit manufacturer. The real-time PCR reaction was performed according to the thermal cycling protocol recommended by the kit manufacturer on a 7500 Fast Real-Time PCR system (Applied Biosystems).

HIV-1 proviral DNA copy numbers per 10^6 PBMC were calculated using the assumption, that 1 µg of human DNA is equivalent of 150,000 genomes.

2.6. Droplet Digital PCR based quantification of HIV-1 proviral DNA

DNA was purified using the DNA Micro Kit (Qiagen), or alternatively DNAzol reagent (Sigma-Aldrich), according to the manufacturers' instructions. Proviral DNA loads were quantified using the QX200 Droplet Digital PCR (ddPCR) platform from Bio-Rad.

For cell-associated HIV DNA Analysis, primers and a 6-FAM-labeled fluorescent probe described for quantitative real-time PCR ("Generic HIV DNA Cell", Biocentric) [277] were used. To quantify HIV genomes/cell, the copy numbers of the single copy gene RPP30 was determined in parallel (PrimePCR ddPCR Copy Number Assay RPP30, HEX, BioRad). The mastermix/well included the following: 2x ddPCR Super-

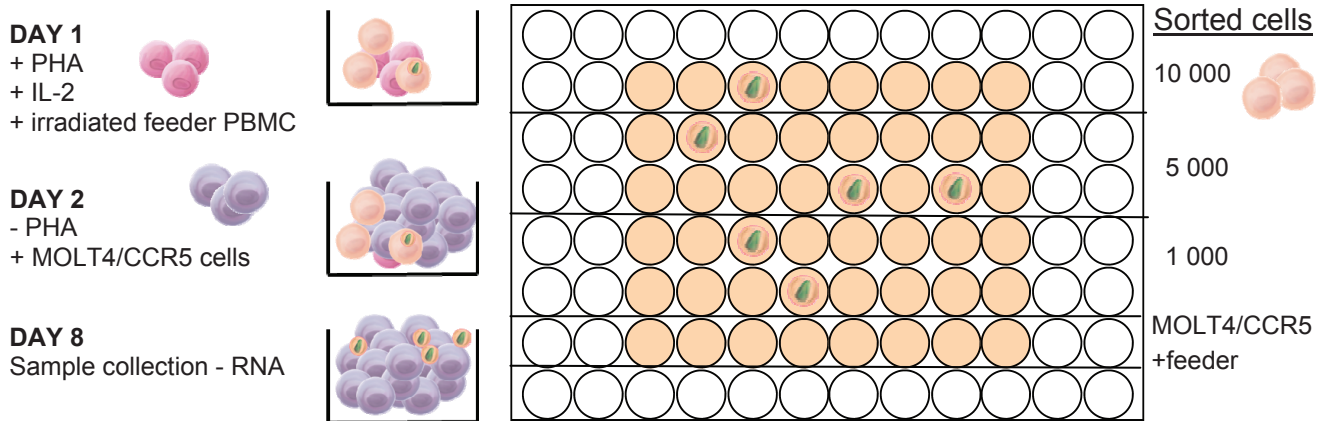
mix for Probes (noUTP) (BioRad) 1:2, HIV-LTR forward and reverse primers as well as the HIV-LTR probe (400 nM each), RPP30 Copy Number Assay (900 nM primers/250 nM probe) and maximum 250 ng template DNA, filled up to 20 ul total volume with RNase free water. Samples were assayed in quadruplicate. “No-template” controls (NTC) containing water instead of template DNA were included in quadruplicate for all assays. Subsequently, this mastermix was used for oil droplet generation using a QX200 Droplet Generator, according to the manufacturer’s instructions. The droplets were transferred into 96 well twin.tec PCR Plates (Eppendorf), the plates were heat-sealed with tin foil and placed in a thermal cycler with a 103 °C heated lid (Analytik Jena). The thermal cycling protocol was the following: 1x (95°C 10 min), 40x(94° C 30 sec – 56.2°C 1 min), 1x(98°C 10 min), 4°C (hold), with a 2°C/sec ramping rate. After thermal cycling, plates were placed into the QX200 ddPCR Plate Reader, and droplets were analysed using QuantaSoft software, version 1.7.4.

For analysis in experiments on sorted CD4+ T cell subsets, amplitude and cluster data were exported from the QuantaSoft program, and fluorescence thresholds for ddPCR quantification were calculated using the ddPCRquant script in R Studio software, based on extreme value theory analysis of droplet fluorescence values in no template control (NTC) wells [322], to provide an objective cut-off with maximum sensitivity (**Figure 3**).

2.7. Quantitative viral outgrowth assay

For the quantification of the replication competent reservoir, cells were plated according to subsets in 96 well round bottom plates in dilutions of 20,000 – 1,000. On average, 8-16 replicates per dilution level could be made. The cells were activated with a combination of phytohaemagglutinin (PHA, 2.5 µg/ml) (Sigma-Aldrich), T cell growth factor (2%) [278,279] and gamma irradiated PBMCs from healthy donors (150,000 – 200,000 /well) as described [253,280] and cultured in the presence of 20 U/ml IL-2. Af-

A



B

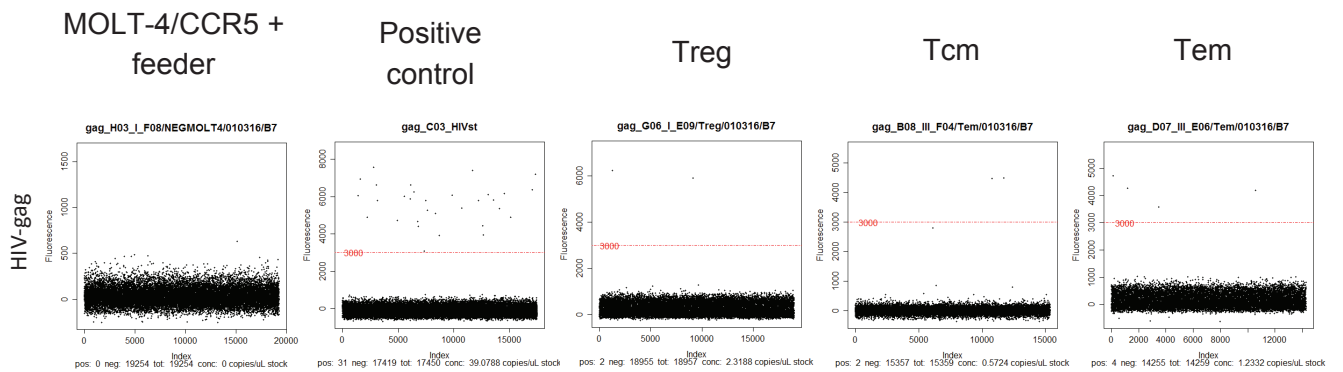


Figure 4. Viral outgrowth assay using an RNA readout detected by ddPCR. (A) Schematic depicting the viral outgrowth assay. **(B)** Representative ddPCR dot-plots showing detection of HIV-gag in positive supernatant samples from different CD4+ subsets of the same donor, along with a negative control sample from the same experiment. Thresholding was performed manually, based on the fluorescence values of the positive control HIV DNA template containing PCR well, to provide a strict and specific cut-off for positive/negative discrimination.

ter 24 hours, PHA containing supernatants were washed off, and 85,000 cycling MOLT-4/CCR5 cells were added to all wells to propagate any infectious HIV [253,280,281]. Wells with parallel cultured feeder PBMC and MOLT4/CCR5 cells, without patient cell input were run as negative control. On day eight of culture, cell-free culture supernatants were harvested. A schematic overview of the viral outgrowth assay is provided in **Figure 4A**.

Supernatants were preserved at -80°C mixed with RNazol reagent (Sigma-Aldrich), and RNA was prepared according to the manufacturer's instructions at a later time point. RNA was reverse transcribed to cDNA using M-MLV Reverse Transcriptase (Promega), according to the manufacturer's instructions.

Subsequently, ddPCR was performed to detect the presence of HIV-*gag* RNA in the supernatant. The following primers and fluorescent probe were used: gag-F (5'-ATCAATGAGGAAGCTGCAGAA-3'), gag-R (5'-GATAGGTGGATTATGTGTCAT-3') gag-probe (5'-(FAM)-ATTGCACCAGGCCAGATGAGAGAA-(BHQ1)-3'), adapted from [282]. An RNA readout was selected, based on increased sensitivity and shorter culture times according to [281]. As a positive control, HIV DNA templates prepared from single integrated proviral HIV carrying cell lines (8E5, alternatively HIVisB2, our own clone) were used [283]. The ddPCR workflow was the same as described above. The mastermix/well included the following: 2x ddPCR Supermix for Probes (noUTP) (BioRad) 1:2, gag-F (1 µM), gag-R (1 µM), gag-probe (250 nM), template cDNA 5 ul, filled up to 20 ul total volume with RNase free water. Fluorescence threshold determination for ddPCR was carried out manually based on fluorescence of template positive droplets in the positive control, to provide maximum specificity for HIV-*gag*, minimize the chance of false positives and provide clear cut-offs for positive/negative discrimination (**Figure 4B**). False positives were not seen in any of the 40 negative control wells across the six experiments presented, or in any of the preliminary experiments while establishing this assay. Infectious units per million cells (IUPM) were calculated based on frequency of wells positive for viral outgrowth at different dilution levels, using ELDA

software [253,280,284,285].

2.8. Analysis of cellular proliferation and activation

PBMC of a healthy donor were stained with the Cell Proliferation Dye eFluor 450 (eBioscience) according to the manufacturer's instructions, and then sorted as CD4⁺ T cell subsets Treg, Tcm and Tem as described before. The purified subpopulations were activated with PHA and gamma-irradiated allogeneic healthy donor PBMC as described for the viral outgrowth assay. Cellular samples were taken at 48 hours, 96 hours and 168 hours after sort. The samples were stained with Live/Dead Near-Infrared Dead Cell Stain Kit (1:100, Life Technologies), and a panel of fluorescently labeled antibodies against CD69 (APC, clone FN50, 10:100), HLA-DR (PerCP/Cy5.5, clone L243, 2:100) and CD25 (PE/Dazzle™594, clone M-A251, 1:100), all purchased from BioLegend. The staining, fixing and analysis steps were the same as described above. Discrimination of sorted CD4⁺ T cell subset cells from feeder PBMC by flow cytometry was possible based on labeling by the cell proliferation dye, which was still detectable after 168 hours post-sort.

3. Results

3.1. Immune phenotypic analysis of the Th17 and regulatory T cell populations in a cohort of HIV-1 patients with various disease courses

As a first objective, Treg (CD25+CD127lo) and Th17 (IL17+IFN γ) populations in the peripheral blood of HIV infected patients were investigated using two multicolor flow cytometry panels.

For the first cohort in this work, 40 HIV-1 infected patients provided PBMC samples. Patients were classified as viremically suppressed by combined antiretroviral therapy (cART, n=20) and as patients with detectable plasma viremia (viremic, n=20). PBMC samples from healthy, aged-matched donors (control, n=12) served as control. The demographic, immunological and virological characteristics of the cohort are listed in **Table 2**. Viremic HIV-1 patients displayed significantly lower CD4+ T cell counts, and a significantly higher degree of immune activation as measured by the expression of HLA-DR on CD3+ T-cells compared to healthy controls and patients treated by cART (**Table 2**).

Analysis of peripheral blood Treg and Th17 frequencies and absolute counts showed different tendencies for these two subsets. While Treg had significantly higher frequencies in the CD4+ T cell population of viremic HIV patients as compared with controls and cART (p=0.0015, **Figure 5A**), the frequencies of Th17 cells appeared to be more stable (**Figure 5B**). Furthermore, absolute counts of these two subsets were calculated based on whole blood CD4+ T cell counts. The absolute counts of Treg were not statistically significantly different in viremic patients as compared with controls and cART (**Figure 5C**). In contrast, significantly lower absolute counts for Th17 cells were detected in the peripheral blood of viremic patients, and the numbers seemed to be restored to healthy levels by cART (**Figure 5D**). When the ratio of Th17/Treg frequencies between the groups was compared, no significant difference was found in viremic

| Parameter | Healthy control | cART | Viremic |
|--|-----------------------|-----------------------|-------------------------------------|
| N | 12 | 20 | 20 |
| Female/male | 3/7 | 4/16 | 2/18 |
| Age (years) | 44.75 (\pm 3.871) | 45.85 (\pm 3.254) | 39.65 (\pm 2.857) |
| Range | 25 – 69 | 23 – 77 | 26 – 76 |
| CD4+ count (cells/ μ l of whole blood) | 1045 (\pm 153.3) | 768.1 (\pm 55.44) | 408.8 (\pm 50.36) ^{a,b} |
| Range | 590 – 2516 | 214 – 1173 | 48 – 808 |
| Plasma viral load (copies/ml plasma) | n/a | < 50 | 224 695 (\pm 71 603) |
| Range | | | 200 – 1 212 000 |
| Expression of HLA-DR on T-cells (%) | 3.996 (\pm 0.5179) | 9.828 (\pm 1.469) | 27.68 (\pm 2.850) ^{a,c} |
| Range | 2.15 – 9.78 | 2.3 – 24.4 | 10.8 – 55 |
| Time on treatment (years) | n/a | 7.455 (\pm 0.9363) | 0.4250 (\pm 0.2190) ^c |
| Range | | 1.2 – 15 | 0 – 3.8 |

Table 2. Demographic and clinical data of the study groups. Values represent means (\pm SEM) and ranges (minimum-maximum). ^ap<0.001 against control, ^bp<0.01 against cART, ^cp<0.001 against cART as determined by one-way ANOVA followed by Bonferroni's Multiple Comparisons test or χ^2 test for differences in female/male ratio.

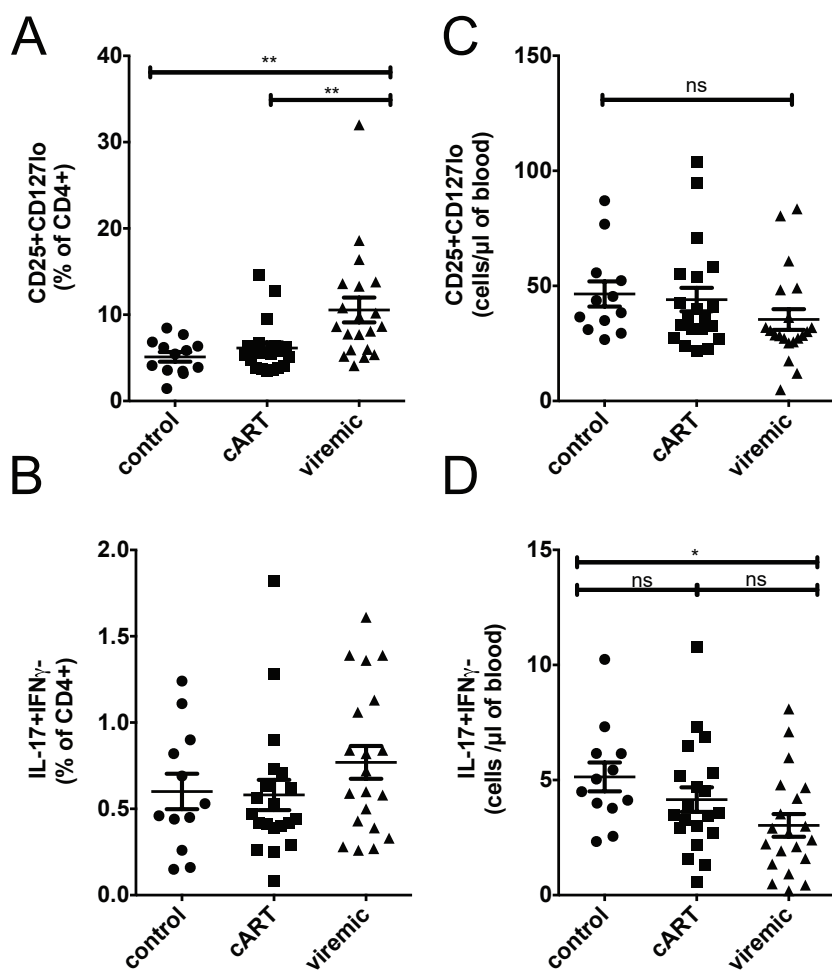


Figure 5. Treg and Th17 frequencies and absolute counts in peripheral blood. PBMC samples of HIV patients (treated, cART n=20 and viremic n=20) as well as healthy donors (control, n=12) were stained by fluorescently labeled antibodies. Treg were identified as CD25+CD127^{lo}. Th17 were identified as CD45RO+IL-17+IFN γ ⁻ after stimulation with PMA and Ionomycin. **(A)** Frequencies of Treg and **(B)** Th17 cells within the CD4⁺ live lymphocytes, and absolute counts of **(C)** Treg and **(D)** Th17 cells calculated based on whole blood CD4⁺ counts. Groups were compared using one-way ANOVA and Bonferroni's Multiple Comparisons Test. *p<0.05, **p<0.01, ns means not statistically significant.

patients or cART as compared to control (not shown).

Next, correlations between Treg and Th17 frequencies and clinical markers of HIV disease progression in all HIV infected patients of the cohort (n=40) were evaluated. The frequencies within the CD4+ T cell population of both Treg (**Figure 6A**) and Th17 cells (**Figure 6B**) correlated inversely with full blood absolute CD4+ T cell counts, but the correlation in the case of Treg was stronger than for Th17 ($p=0.0001$ and $p=0.0449$ respectively, **Figure 6A** and **B**). The absolute counts of both subsets seemed to be strongly linked to absolute CD4+ T cell counts (**Figure 6C** and **D**), with strong correlations in the case of Treg ($p=0.0002$), as well as Th17 cells ($p=0.0003$).

A marked difference could be observed in the behavior of these two subsets in relation to plasma viral loads in viremic HIV patients (n=20). The frequencies of Treg within the CD4+ T cell population correlated highly significantly with plasma viral loads ($p=0.0001$) (**Figure 6E**), but absolute numbers did not (not shown). In contrast, Th17 frequencies did not correlate with plasma viral loads (not shown), while Th17 absolute counts exhibited significant negative correlation with the concentration of RNA copies of HIV in the plasma ($p=0.0319$, **Figure 6F**).

The frequencies of peripheral blood Th17 and Treg populations were longitudinally investigated in four patients, from whom cryopreserved PBMC samples from before initiation of cART, as well as follow-up samples spanning eight years of therapy were available. All of the patients exhibited a significant drop in plasma viremia within the first year of therapy, which then remained below 100 copies/ml in all cases (**Figure 7A**). All but one of the patients had achieved restoration of CD4+ T cell numbers to above 500 cells/ μ l within five years of therapy (**Figure 7B**). In the case of one patient, a limiting number of cryopreserved PBMC from before treatment were available, and only Th17 were analyzed at baseline. No significant changes were observed for Treg frequencies and absolute counts in this small longitudinal cohort (**Figure 7C** and **D**). In contrast, a significant stepwise restoration of Th17 cells could be detected in the peripheral blood of the four HIV patients analyzed longitudinally at one, five and eight years of

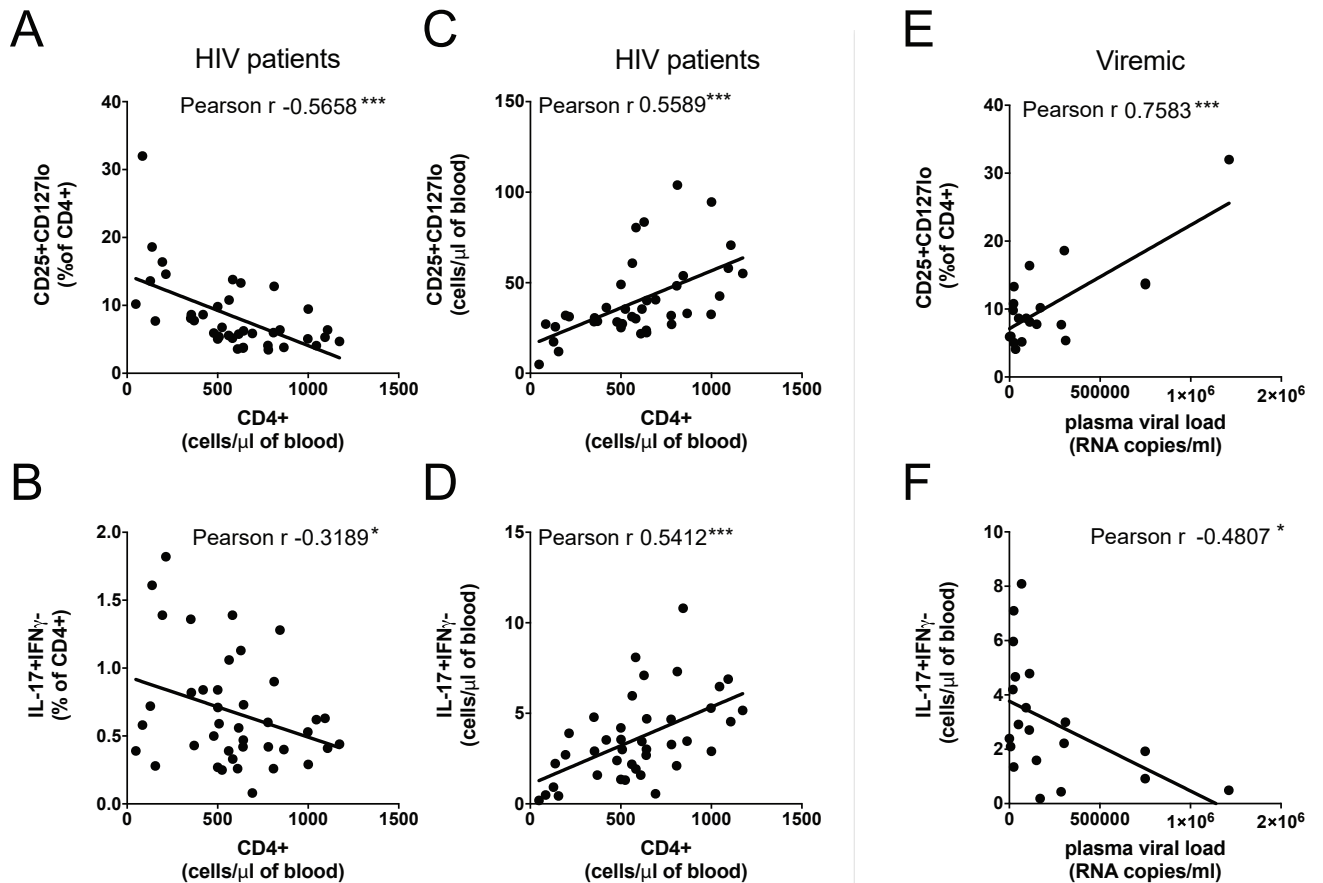


Figure 6. Correlation of Treg and Th17 frequencies and absolute counts with clinical markers of disease progression. (A) Correlation of CD25+CD127^{lo} Treg frequencies and (B) correlation of IL-17+IFN γ - Th17 frequencies with clinical full blood CD4+ counts in all HIV infected patients in the cohort (n=40). (C) Correlation of Treg and (D) correlation of Th17 absolute counts with clinical CD4+ counts. (E) Correlation of Treg frequencies and (F) correlation of Th17 absolute counts with clinical plasma viral loads in patients with viremia (n=20). Pearson correlation “r” values are shown in the graphs. *p<0.05, ***p<0.001.

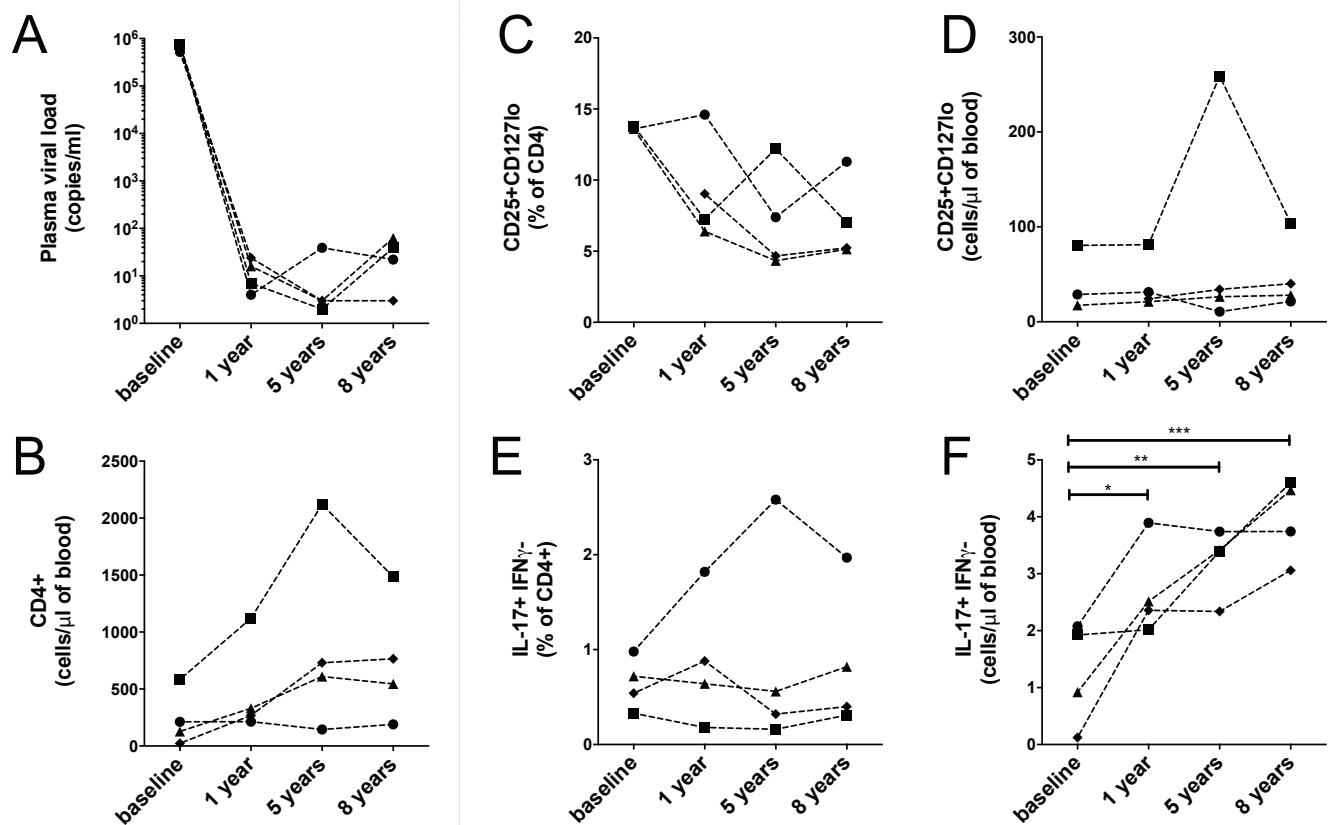


Figure 7. Longitudinal follow up of Treg and Th17 populations in the peripheral blood of 4 HIV infected patients during 8 years of cART. (A) Plasma viral loads and (B) peripheral blood clinical CD4+ counts. (C) CD25+CD127lo Treg frequencies within the CD4+ population and (D) Treg absolute counts in peripheral blood. (E) IL-17+IFN γ - Th17 frequencies within the CD4+ population and (F) Th17 absolute counts in peripheral blood. Baseline means before initiation of cART. All patients were treated continuously for 8 years. Sampling timepoints after initiation of cART are shown on the x axis, and are approximate. Different shapes represent different donors, and are consequent across all graphs. Time points were compared by repeated measures ANOVA, followed by Bonferroni's Multiple Comparisons Test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

therapy (**Figure 7F**). Th17 frequencies within the CD4⁺ T cell population appeared to be stable in the three patients who responded to cART with normalization of CD4⁺ T cell counts (**Figure 7E**). In contrast, a longitudinal increase in Th17 frequencies in the non-responder patient could be observed, possibly contributing to the restoration of Th17 absolute counts despite CD4⁺ T cell counts that remained low after eight years of cART in this patient (**Figure 7E and F**).

3.2. Comparative analysis of Th17 cells identified by surface markers versus intracellular cytokine staining

The data presented in the following section has been published previously [276].

Analysis of the Th17 subset as a potential reservoir of HIV requires live sorting of Th17 cells with considerable purity. Because surface marker characterization of Th17 cells, based on the expression of chemokine receptors and CD161 is controversial in the literature, a direct, parallel comparison of the frequency of Th17 cells was performed using two methods in healthy controls and HIV patients. Since *in vitro* stimulation by PMA and ionomycin significantly alters the expression of a variety of cell surface antigens, performing the staining for the surface markers of Th17 cells and the IL-17 intracellular staining at the same time was not possible. Therefore, clinical PBMC samples were simultaneously analyzed in parallel by 1.) a surface staining protocol to identify CD4⁺CD45RO⁺CXCR3⁻CCR6⁺CCR4⁺CD161⁺ cells [156,198,200,252,265] and by 2.) an intracellular staining protocol to detect CD4⁺CD45RO⁺IL-17⁺IFN γ ⁻ functional Th17 cells [78–80,94,264,286]. Representative plots depicting the two approaches are shown in **Figure 8**.

The cohort, and the study groups were introduced in the previous section (**Table 2**). First, the Th17 frequencies determined by the two methods were compared. Throughout all patient groups, irrespective of their HIV status, the relative frequency of Th17 cells as estimated by the CD4⁺CD45RO⁺CXCR3⁻CCR6⁺CCR4⁺CD161⁺ phenotype

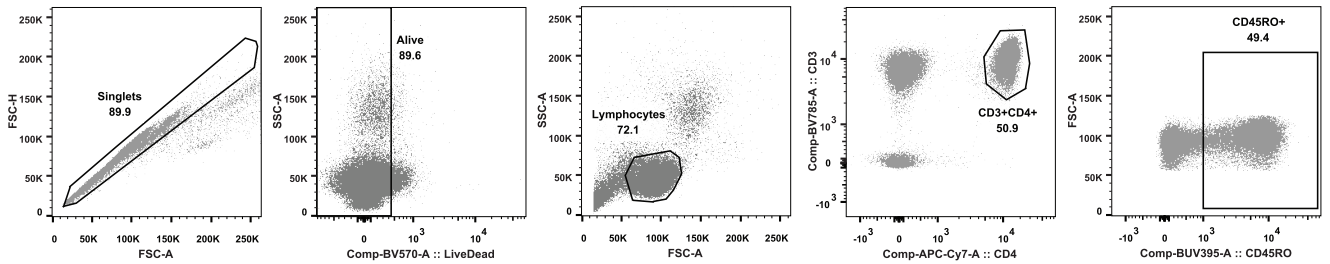
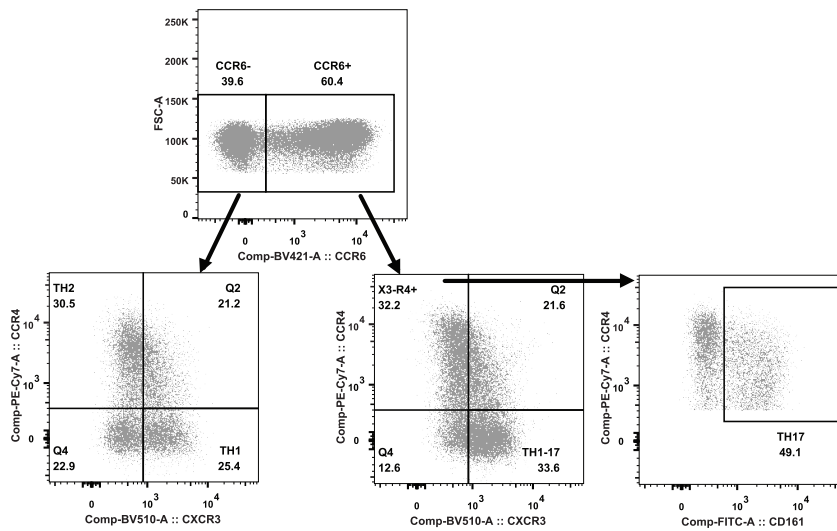
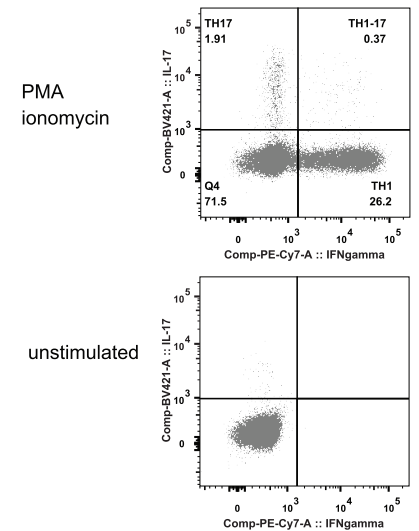
A**B****C**

Figure 8. Representative dot plots from a healthy donor depicting the two approaches of Th17 identification. (A) With both approaches, Th17 gating was performed on live CD3+CD4+CD45RO+ lymphocytes. **(B)** Surface staining: Th17 cells were defined as CCR6+CXCR3-CCR4+CD161+ cells as displayed above. **(C)** Intracellular cytokine staining: Th17 cells were defined as cells positive for IL-17A and negative for IFN γ , to distinguish from IL-17A and IFN γ double positive TH1/17 cells and IFN γ single positive Th1 cells. The unstimulated control is shown below. Names of gates are shown, along with numbers representing percentage of parent gate values.

of the total CD4⁺ T cell population was higher than the Th17 frequency measured by ICS (**Figure 9A**). The fold-difference of the means of both frequencies was 6.73 for healthy controls, 7.13 for the cART group and 6.53 for viremic patients (**Figure 9A**). Analysis of the absolute counts of Th17 cells showed similar differences between the two methods (**Figure 9B**).

In order to see whether the two phenotypes principally identify a common cell type in all groups, the correlation between the frequencies of Th17 cells as estimated by the CD4⁺CD45RO⁺CXCR3⁻CCR6⁺CCR4⁺CD161⁺ phenotype and by IL-17A ICS was analyzed. As expected, there was a significant correlation between the two phenotypes in all groups (healthy controls Pearson $r=0.6908$, $p=0.0129$; viremic HIV patients Pearson $r=0.5373$, $p=0.0146$; cART patient group Pearson $r=0.8270$; $p<0.0001$ - **Figure 9C**).

In healthy donors, absolute Th17 numbers did not correlate with CD4⁺ T cell counts and had high individual variations using both methods of identification (**Figure 9D**). In contrast, the absolute counts of IL-17-IFN γ ⁺ Th1 cells showed a significant correlation with CD4⁺ T cell counts in healthy donors (data not shown), suggesting a lower inter-individual variability of the Th1 subset.

The absolute numbers of IL-17+IFN γ ⁻ cells correlated with CD4⁺ T cell counts and inversely correlated with plasma viral loads, as described in the previous section. Both of these correlations held true using the CXCR3-CCR6+CCR4+CD161+ phenotype (**Figure 9E and F**).

3.3. Adaptation of a commercially available quantitative PCR assay for use with Droplet Digital PCR for the quantification of proviral HIV-1 DNA sequences in clinical samples

The next aim of this work was to investigate the potential use of Droplet Digital PCR (ddPCR) for sensitive and accurate quantification of relative cell-associated HIV proviral DNA burden in clinical samples. For this, the commercially available “HIV Generic Cell”

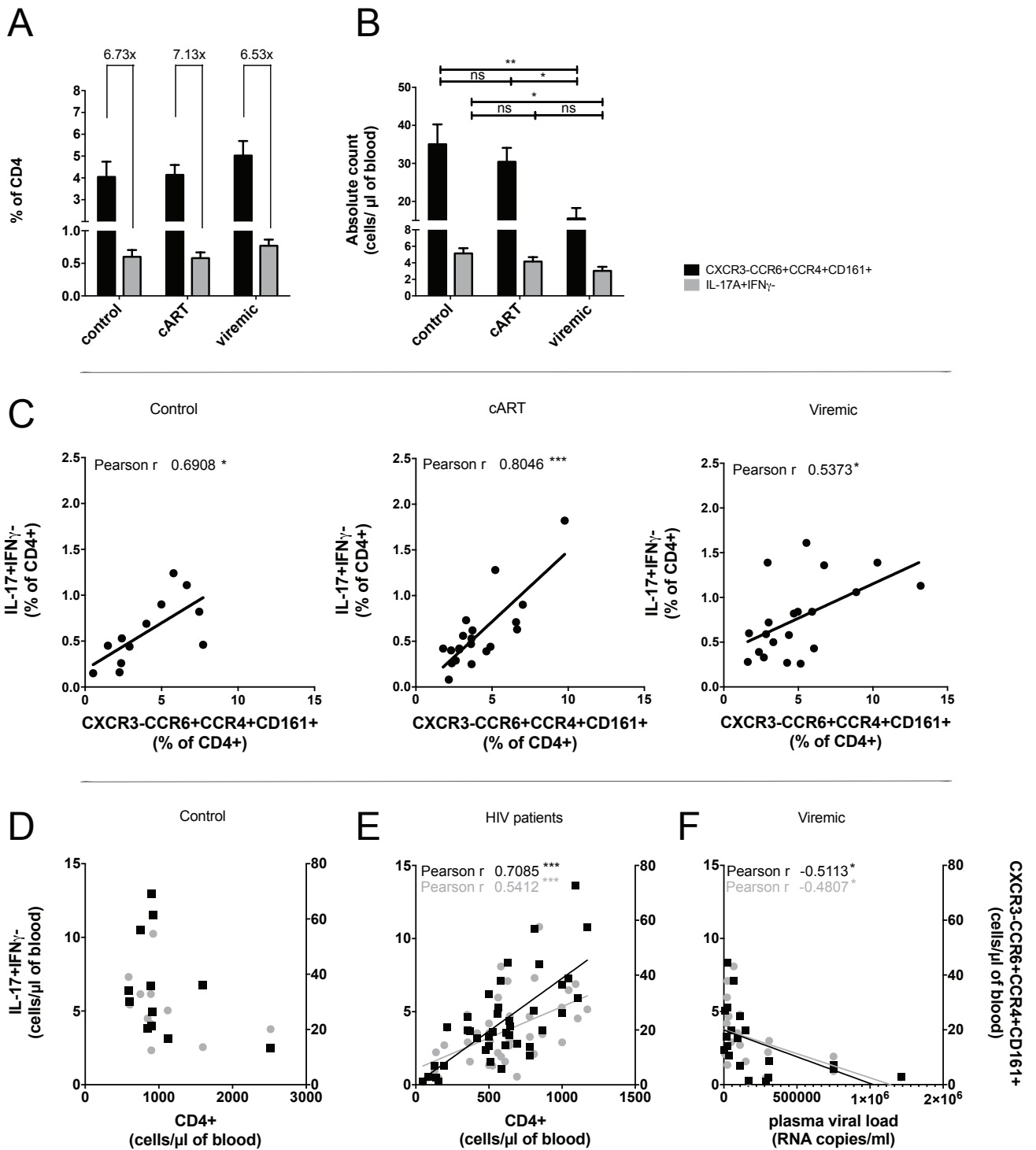


Figure 9. Comparative analysis of the Th17 cell population in HIV disease with two different methods. PBMC samples of HIV patients (treated, cART n=20 and viremic n=20) as well as healthy donors (control, n=12) were stained for Th17 cells as described for gating strategy. **(A)** Frequency and **(B)** absolute counts of Th17 cells identified by both methods. Connecting lines show the fold difference between the frequencies obtained by surface staining and IL-17A ICS. **(C)** Correlation of the two methods in determining the frequencies of Th17 cells by study group. **(D)** Correlations between absolute numbers of Th17 cells identified by both methods and clinical CD4+ counts in healthy controls and **(E)** all HIV patients (cART and viremic) and with **(F)** plasma viral loads of viremic patients. Groups were compared with one-way ANOVA and Bonferroni's Multiple Comparisons Test. Correlations were analyzed using Pearson's Correlation Test, Pearson r values are shown on the graphs. Columns show means and bars indicate SEM. Color codes for different phenotypes are shown in the figure. *p < 0.05, **p < 0.01, ***p < 0.001.

kit for quantitative real-time PCR based quantification of cell-associated proviral HIV DNA (Biocentric, France) was selected and adapted for use with the ddPCR platform. This assay amplifies a conserved consensus region in the 5'LTR of HIV [262,277,287–289], and is recommended for use in clinical studies by the French National Agency for AIDS Research (ANRS).

PBMC of seven HIV infected patients, who started treatment during the acute phase of the disease, were available for this study (**Table 3**, patients marked with asterisk). Baseline samples were taken before initiation of cART, or in the very early phase of treatment, before plasma viremia was suppressed. Furthermore, follow up PBMC samples were available from these patients, taken at visits one to four years after initiation of therapy. Six baseline and six follow up samples were selected for comparison of ddPCR and qPCR based quantification. The hypothesis was that samples taken in the acute phase of HIV infection were likely to have a high proviral HIV burden in PBMC, whereas considerably lower proviral DNA loads were expected in samples after cART. Thus, the performance of qPCR and ddPCR could be compared across a wide range of proviral HIV DNA loads.

First, the absolute quantification of HIV-LTR copy numbers in the stock solution of DNA prepared from PBMC was compared. A highly significant correlation between qPCR and ddPCR ($p < 0.0001$), with a high degree of linearity ($R^2 = 0.9684$) was observed (**Figure 10A**). The Bland-Altman analysis revealed an average difference of $0.1104 \pm 0.364 \log_{10}$ (ddPCR-qPCR) (**Figure 10B**), which is within the accepted assay variability of $0.5 \log_{10}$ in similar studies [290,291].

Next, the performance of these two assays in quantifying relative cellular proviral DNA copy numbers was evaluated. The correlation was similarly significant ($p < 0.0001$) in this case (**Figure 10C**). Linearity was slightly less ($R^2 = 0.9080$), and the average difference was $-0.04366 \pm 0.50014 \log_{10}$ (**Figure 10D**). In light of the high degree of assay concordance in absolute HIV-LTR quantification, the decreased linearity and higher standard deviation was probably due to the fact that the two assays apply a differ-

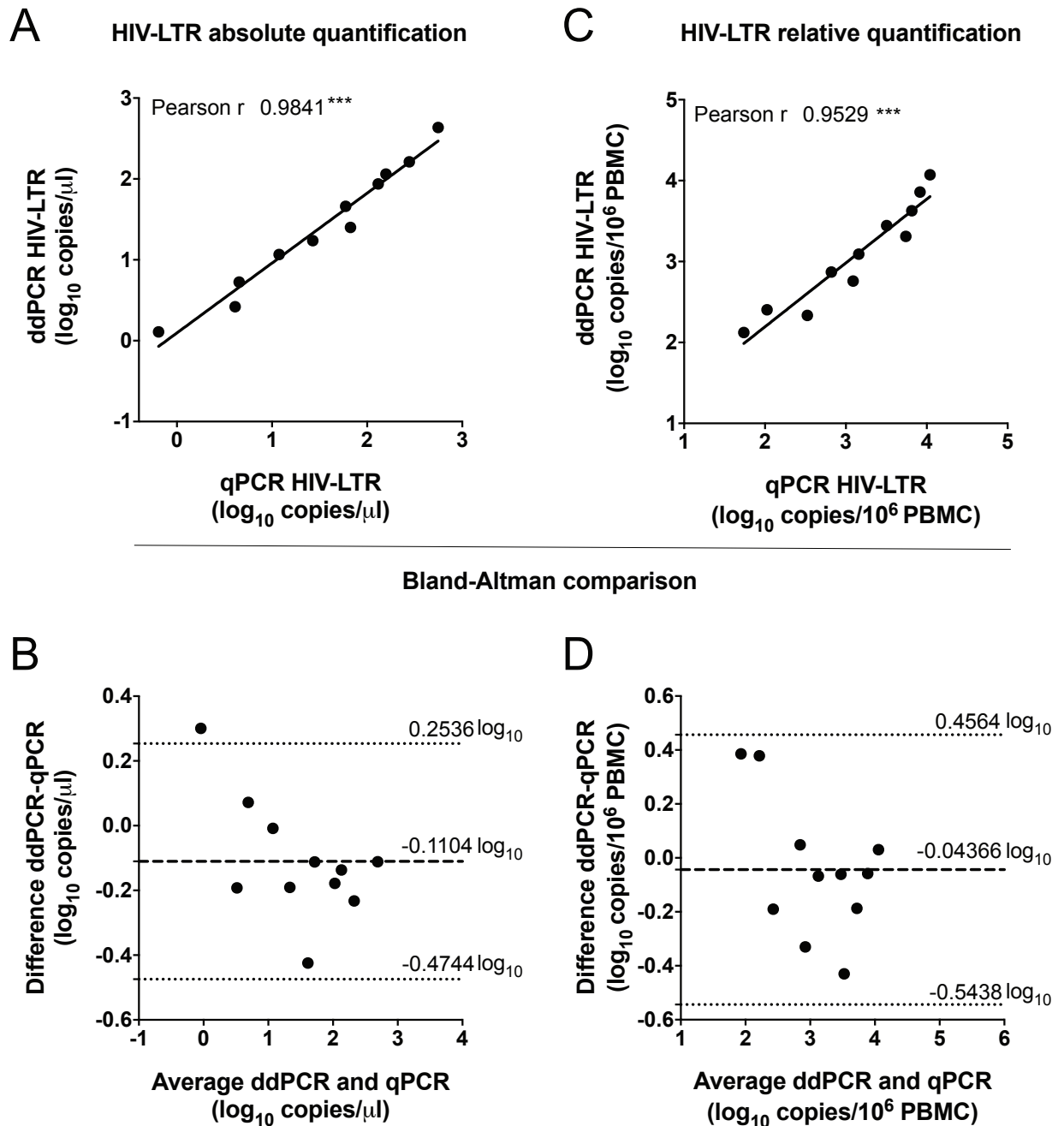


Figure 10. Quantification of HIV-LTR sequences in clinical PBMC samples using qPCR versus ddPCR. The “Generic HIV DNA Cell” kit for quantitative real-time PCR (qPCR) was adapted for use with duplex Droplet Digital PCR (ddPCR) with tandem quantification of the genomic single copy gene RRP30. HIV-LTR sequences were quantified in DNA from 12 clinical PBMC samples by qPCR according to the manufacturer’s instructions, and by the novel ddPCR-based approach. **(A)** Correlation of absolute concentrations as determined by qPCR and ddPCR. **(B)** Correlation of relative HIV-LTR copy numbers in 10⁶ PBMC as determined by qPCR and ddPCR. For qPCR, genomic input was estimated based on spectrophotometrically determined DNA quantity, for ddPCR it was determined based on copy numbers of RPP30. **(C)** Bland-Altman plots comparing qPCR and ddPCR measurement of absolute and **(D)** relative HIV-LTR copy numbers using qPCR and ddPCR. Correlations were analyzed using Pearson’s Correlation Test, Pearson r values for correlation and mean differences (bias) and 95% Limits of Agreement for Bland-Altman comparison are shown on the graphs. ***p < 0.001.

ent approach when quantifying genomic input. While the commercial assay requires determination of genome count based on spectrophotometric measurement of DNA concentration in the sample, the ddPCR assay measures a genomic single copy gene (RPP30) in parallel, thereby allowing for direct determination of genome count within the same PCR reaction.

3.4. Evaluation of proviral DNA burden in PBMC of patients with early or late initiation of combined antiretroviral therapy

Early treatment has been shown to limit viral reservoirs in the SIV model [292], as well as human studies [261,293,294]. ddPCR was used to compare the dynamics of proviral DNA decay in a cohort involving patients who started cART in the acute phase of disease (**Table 3A**), as well as patients who started cART with established chronic HIV infection (**Table 3B**). Patients with acute infection were defined by either a negative HIV antibody response at the time of diagnosis by PCR, or by seroconversion within six months of a documented negative HIV antibody test, who had begun cART within six months of diagnosis. Patients with chronic infection were patients who were not diagnosed in the acute phase, and had begun treatment in the chronic phase of the disease (**Table 3B**).

Development of proviral DNA loads in PBMC in the study groups is shown in **Figure 11**. Paired samples for seven donors were available at baseline and after one year of therapy for both the acute and chronic treatment groups. In both groups, the changes in proviral DNA copies in PBMC were not statistically significant after one year of treatment (acute $p=0.1228$, chronic $p=0.4589$ by two-way repeated measures ANOVA, **Figure 11**). In addition, no significant difference was observed in proviral DNA copies in PBMC between the acute and chronic treatment groups at baseline or after one year of cART ($n=7$ paired samples for acute and chronic, baseline $p=0.6636$, one year $p=0.9986$ by two way repeated measures ANOVA, not shown).

A

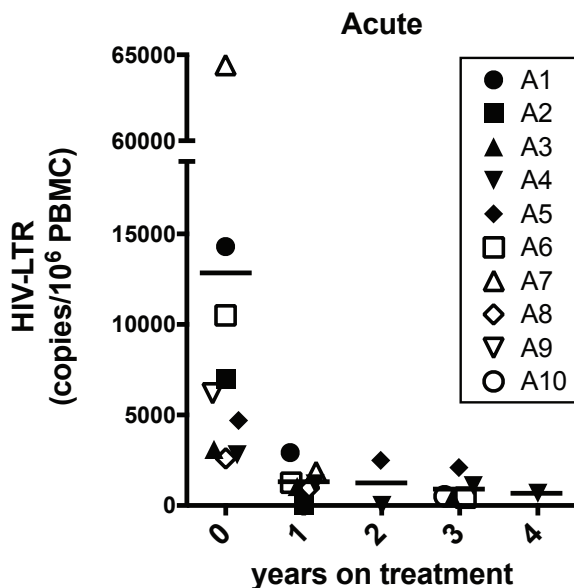
| Acute cohort | Age | Plasma viral load (RNA copies/ml) | CD4+ count (cells/ μ l of blood) |
|--------------|-----|-----------------------------------|--------------------------------------|
| A1 # | 32 | 3 300 000 | 203 |
| A2 # | 23 | 10 000 000 | 108 |
| A3 # | 47 | 2 700 | 189 |
| A4 # | 39 | 1 700 | 325 |
| A5 # | 53 | 140 000 | 548 |
| A6 | 32 | 15 000 000 | 113 |
| A7 | 26 | 3 300 000 | 253 |
| A8 | 33 | 180 000 | 349 |
| A9 # | 21 | 215 000 | 278 |
| A10 # | 31 | no baseline available | |

B

| Chronic cohort | Age | Plasma viral load (RNA copies/ml) | CD4+ count (cells/ μ l of blood) |
|----------------|-----|-----------------------------------|--------------------------------------|
| C1 | 39 | 42 000 | 188 |
| C2 | 65 | 40 000 | 241 |
| C3 | 74 | 46 000 | 461 |
| C4 | 28 | 112 000 | 352 |
| C5 | 34 | 75 000 | 392 |
| C6 | 28 | 10 000 | 358 |
| C7 | 53 | 75 000 | 214 |
| C8 | 72 | 21 000 | 177 |
| C9 | 27 | 13 000 | 384 |

Table 3. Showing characteristics of the cohort for determination of proviral DNA loads in PBMC by quantitative real time PCR (qPCR), and Digital Droplet PCR (ddPCR). **(A) Acute cohort.** Cohort of patients identified as acutely HIV infected at the time of the baseline sample by the treating physician, and have immediately begun therapy. **(B) Chronic cohort.** Cohort of patients who presented with chronic HIV infection, and subsequently began cART. Age, plasma viral loads and CD4+ counts at the time of the baseline sample are shown. # patients who were included in the comparative analysis by qPCR and ddPCR in Figure 10.

A



B

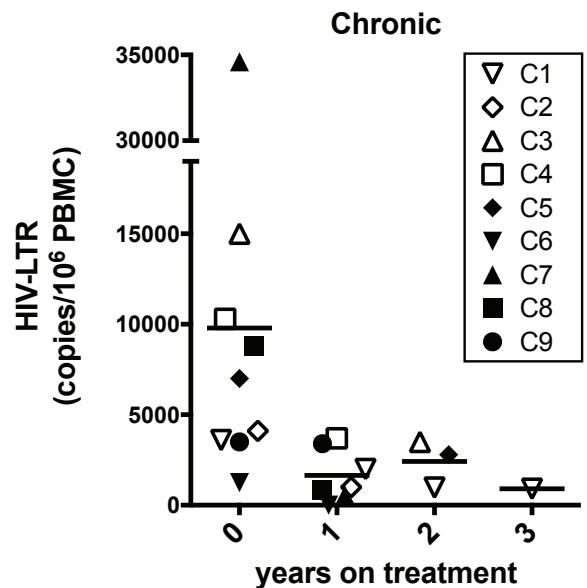


Figure 11. HIV infected patients were followed up for 1-4 years for proviral HIV-1 DNA burden in PBMC using ddPCR. **(A) Acute cohort.** **(B) Chronic cohort.** See Table 3 for description of cohorts. Sampling times after cART initiation are shown on the x axis, and are approximate. Bars show mean values at given time point.

3.5. Quantification of proviral DNA loads in different CD4⁺ T cell subsets in a cohort of chronic HIV patients

The original aim of this work was to compare HIV viral reservoirs in Treg and Th17 cells to the proviral burden in Tcm and Tem cells. In previous experiments it had been shown, that surface marker staining is unlikely to yield a pure population of Th17 cells, because on average seven times as many cells express the CXCR3-CCR6+CCR4+CD161⁺ as cells that are IL-17⁺IFN γ ⁻ functional Th17 cells (**Figure 9A**). Thus, sorting of Th17 cells based on surface expression of IL-17 was attempted [295]. This approach, however, did not yield sufficient cell numbers to allow for HIV reservoir studies using the approximately 50 ml blood donations available. As a result, only the HIV reservoir in Treg as compared to Tcm and Tem was investigated in further studies.

Ten patients with chronic HIV infection (mean time since infection 112 months) were enrolled in this study. All patients had received long-term cART (mean treatment time 86.4 months). The minimal time of undetectable plasma viremia (defined as < 50 RNA copies/ml) was two years (mean of 66 months). The clinical characteristics of the cohort are shown in **Table 4**.

CD4⁺ T cell enriched PBMC samples from the HIV infected donors were sorted for CD25⁺CD127^{lo} Treg, CD45RO⁺CCR7⁺ Tcm and CD45RO⁺CCR7⁻ Tem cells.

The differences of proviral DNA load in Treg, Tcm and Tem (n=10) were not statistically significant in this cohort (mean \pm SEM Treg: 7266 \pm 3669, Tcm 3454 \pm 1138, Tem: 6306 \pm 1833 HIV-1 copies/million cells, Friedman test p=0.3159, **Figure 12**). Unsorted CD4⁺ T cells (n=5) had an average of 2211 \pm 741.6 HIV-1 copies/million cells.

The strongest association detected between clinical parameters (**Table 4**) and proviral DNA loads (**Figure 12**) was a significant correlation between peak plasma viremia and HIV-1 copies per million cells in Tcm and Treg (Pearson r 0.8724, p=0.001 and Pearson r 0.8482, p=0.0019, respectively, not shown) but not in Tem (Pearson r: 0.4035, p=0.2476, not shown). No significant correlation could be observed for com-

| ID | age (years) | sex | months infected* | months on cART | months suppressed** | peak viremia*** (RNA copies/ml) | CD4+ nadir*** (cells/ul of blood) | CD4+ count (cells/ul of blood) |
|-------|-----------------|-----|------------------|------------------|---------------------|--|-----------------------------------|--------------------------------|
| E1 | 28 | F | 19 | n/a | n/a | n/a | 266 | 446 |
| A2 ● | 36 | M | 36 | 35 | 32 | 15 000 000 | 113 | 402 |
| B2 ▼ | 38 | M | 58 | 50 | 40 | 2 040 000 | 490 | 725 |
| B3 □ | 29 | M | 95 | 79 | 67 | 330 000 | 198 | 647 |
| B4 ○ | 44 | M | 156 | 151 | 51 | 286 000 | 254 | 671 |
| B5 ▲ | 58 | M | 54 | 27 | 28 | 88 000 | 343 | 765 |
| B6 ▲ | 49 | F | 285 | 232 | 232 | 23 000 | 270 | 565 |
| B7 ▼ | 54 | M | 114 | 62 | 61 | 1 460 000 | 436 | 755 |
| B8 ◇ | 69 | M | 174 | 133 | 85 | 100 000 | 137 | 406 |
| B9 ■ | 44 | M | 84 | 34 | 32 | 4 600 000 | 306 | 772 |
| B10 ◆ | 44 | M | 64 | 61 | 32 | 670 000 | 82 | 361 |
| | 46.5 (±3.66) | | 112 (±23.84) | 86.4 (±20.84) | 66 (±19.37) | 2.46 x 10 ⁶ (±1.46 x 10 ⁶) | 262.9 (±42.84) | 606.9 (±51.47) |

Table 4. Cohort of patients on long-term suppressive combined antiretroviral therapy. Characteristics of the cohort including 1 HIV elite controller (E1) and 10 patients undergoing chronic combined antiretroviral therapy. The bottom row shows mean (±SEM) values. Icons next to patient ID show legend for Figure 12. *earliest confirmed infection, when exact date not available. **time elapsed since last measurable viral load (>50 copies/ml). **highest/lowest values detected at routine check-up.

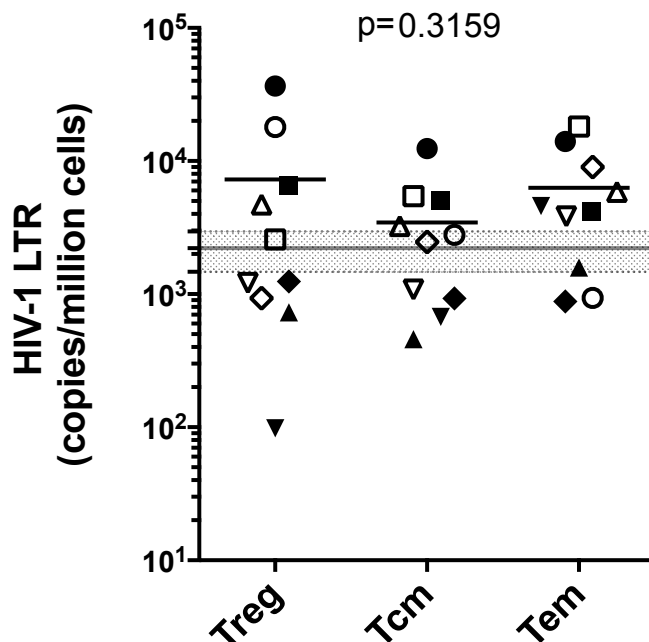


Figure 12. Proviral DNA copy numbers in CD4+ subsets. Proviral HIV-1 DNA copies per million cells for the different sorted subsets as indicated below (n=10). Different donors are marked according to Table 4. Thick grey line showing mean proviral DNA copy number for unsorted CD4+ T cells (n=5), dotted lines showing ± SEM. Groups were compared using the Friedman test, p value is shown above the graph.

parisons with duration of infection, time on treatment, time without detectable viremia, CD4+ T cell nadir or current CD4+ T cell counts. However, statistical power to analyze these correlations was quite limited, due to the small sample size (n=10).

3.6. Quantification of replication competent viral reservoir in different CD4+ T cell subsets in a cohort of chronic HIV patients

To measure the proportion of cells carrying replication competent virus in HIV infected patients on long-term antiretroviral therapy (**Table 4**), it was necessary to ensure that the method for T cell stimulation generally applied in the viral outgrowth assay for CD4+ T cells [210,253,279,280] produces cellular activation to the same extent in all subsets (Treg, Tcm and Tem).

PBMC from a healthy donor were stained with a fluorescent cell proliferation dye, and were then sorted as described above, to obtain purified Treg, Tcm and Tem subsets. Afterwards, cells were activated as discussed, and cell proliferation and activation were assessed based on dilution of the proliferation dye, as well as expression of early activation marker CD69 and late activation markers CD25 and HLA-DR.

Figure 13 shows a representative experiment on *in vitro* activation of the different CD4+ T cell subsets. CD69, a marker for early cell activation was expressed by approximately 80% of cells independent of CD4+ T cell subset phenotype after 48h of activation (**Figure 13A**). Afterwards, CD69 was downregulated, and late activation markers CD25 and HLA-DR were upregulated in all subsets, reaching a combined expression of close to 100% in all subsets (**Figure 13B**). In parallel, cells were dividing, as evidenced by decrease of fluorescence intensity of the cell proliferation dye (**Figure 13C**). By 168h after activation, cells in which cell division had not taken place could no longer be detected in any of the subsets (**Figure 13C**). Thus, an equally effective mitogenic effect of the stimulus, regardless of subset, could be demonstrated. Based on this data, no differences could be observed between CD4+ T cell subsets in their re-

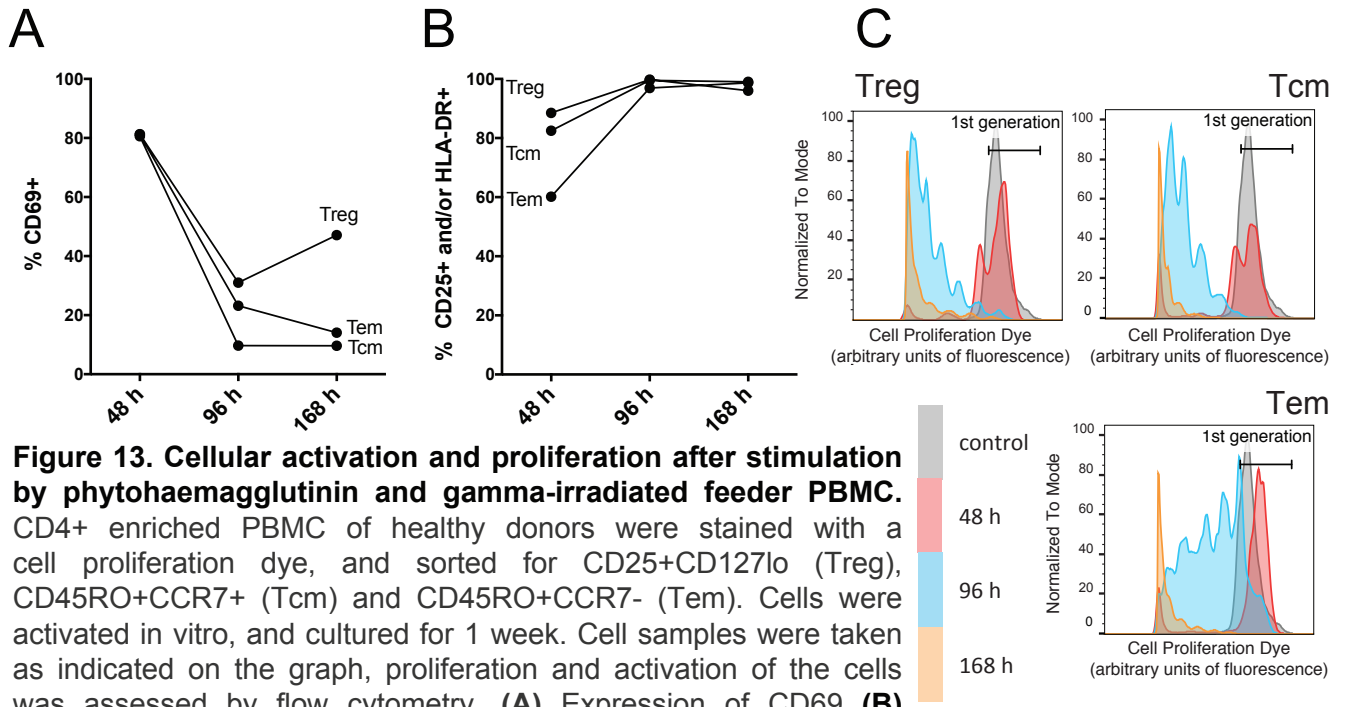


Figure 13. Cellular activation and proliferation after stimulation by phytohemagglutinin and gamma-irradiated feeder PBMC. CD4⁺ enriched PBMC of healthy donors were stained with a cell proliferation dye, and sorted for CD25⁺CD127^{lo} (Treg), CD45RO⁺CCR7⁺ (Tcm) and CD45RO⁺CCR7⁻ (Tem). Cells were activated in vitro, and cultured for 1 week. Cell samples were taken as indicated on the graph, proliferation and activation of the cells was assessed by flow cytometry. **(A)** Expression of CD69 **(B)** expression of CD25 and/or HLA-DR according to subset at the indicated timepoints. **(C)** Cell proliferation was followed by decreasing fluorescence, as the cell proliferation dye is distributed equally among daughter cells. One representative experiment out of two is shown.

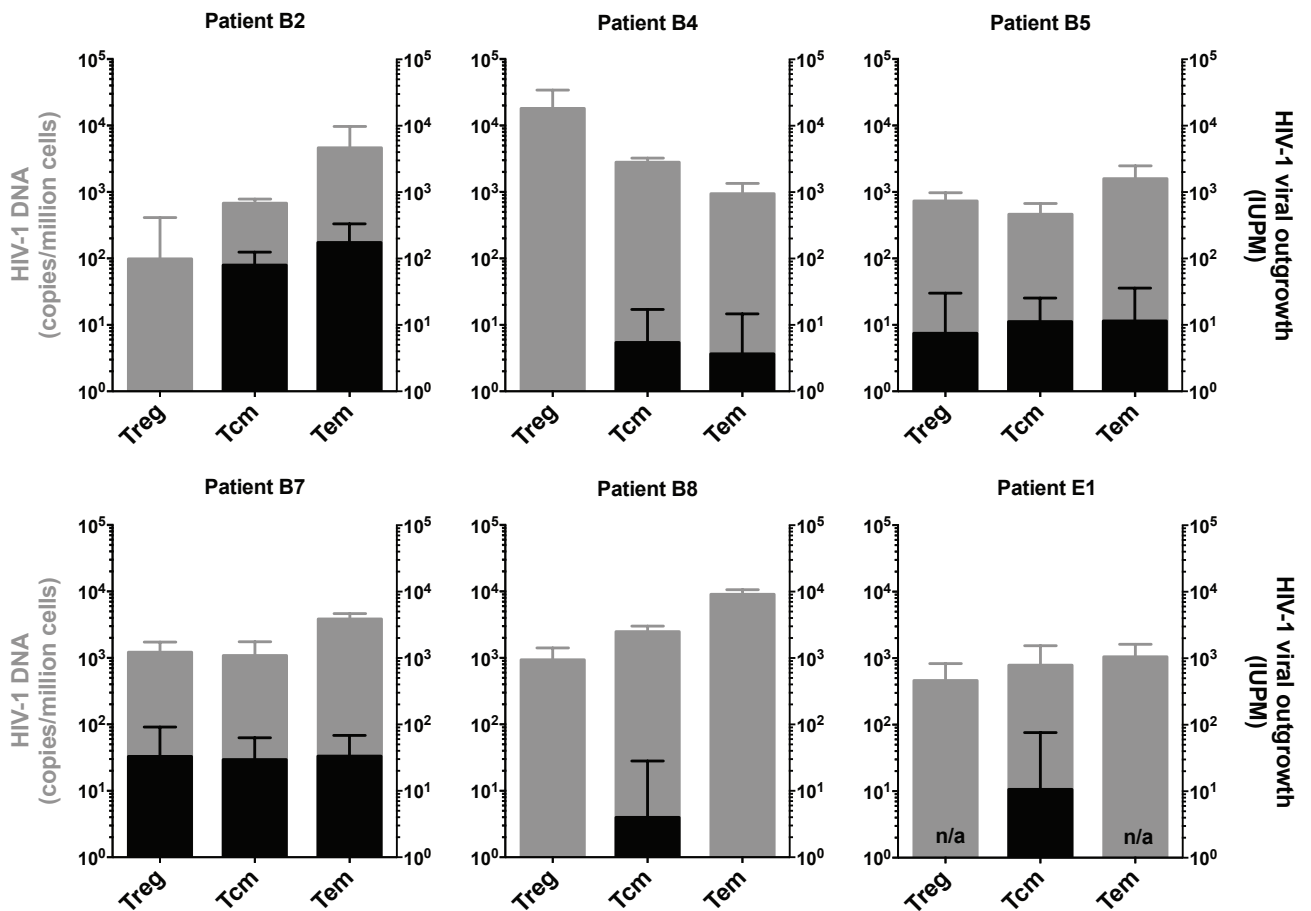


Figure 14. Viral outgrowth assay results. Infectious units per million cells (IUPM) values are shown in black, overlaid on corresponding proviral DNA copy number data in gray, according to patients and CD4⁺ T cell subsets as indicated in the figure. Columns show mean, and bars represent 95% confidence intervals.

response to stimulation by PHA and gamma-irradiated feeder PBMC, and cell activation and proliferation response was similarly complete in Treg, Tcm and Tem.

In sorted CD4⁺ T cell subsets of HIV infected patients on long-term cART, viral outgrowth was detected based on the presence of HIV-*gag* RNA in the culture supernatant eight days after stimulation. Infectious units per million (IUPM) values in Tcm cells were successfully quantified in all cases (n=5), (**Figure 14**). For Patient B8, viral outgrowth could only be detected in the Tcm subset. On average, Tem appeared to harbor the highest amount of replication competent virus, but the differences between Treg, Tcm and Tem were not statistically significant (p=0.1242 with the Friedman test, not shown).

In the case of two donors, IUPM values for Treg cells could be successfully quantified, which is a novel finding of this study (**Figure 14**, Patients B5 and B7). The replication competent reservoir associated with Treg in these individuals was similar to those seen in Tcm and Tem. In three out of five donors investigated, no viral outgrowth was observed from the Treg subset (**Figure 14**, Patients B2, B4 and B8), even though the average number of Treg obtained per sort was not significantly different from that of Tem (not shown). Moreover, general cell activation of Treg was determined to be similarly complete as in the case of Tcm and Tem (**Figure 13**).

In addition to patients on long-term cART, an HIV elite controller (**Figure 14**, patient E1) was also included in this study. Interestingly, even though this patient never developed a plasma viral load measurable by PCR, proviral DNA could be measured in all of the CD4⁺ T cell subsets Treg, Tcm and Tem, and viral outgrowth was measurable in Tcm (the viral outgrowth assay could not be performed for Treg and Tem because of limiting cell numbers).

4. Discussion

4.1. Phenotypic alteration of Th17 and Treg in the course of HIV-1 infection

In the first part of this work, a phenotypic analysis of Th17 and Treg populations was performed in the peripheral blood of HIV-1 infected patients with ongoing virus replication or suppressed viremia due to cART, as well as healthy control subjects.

CD4⁺ T cell depletion is a hallmark of HIV infection, thus, absolute counts of CD4⁺ T cell subsets are generally expected to be also lower in patients with ongoing virus replication. However, relative frequencies of Treg within the CD4⁺ T cell pool have been shown to be expanded in peripheral blood [82,83,296–302] and also in the gut [303,304] in untreated HIV infection, and this was also the case in the present study. This significant relative increase of Treg frequencies might be able to compensate for the HIV-related cell depletion involving all CD4⁺ T cells to some extent, causing relatively stable Treg absolute counts compared to other subsets. To provide an explanation to this phenomenon, the inflammatory state driving Treg proliferation might cause expansion of Treg [82]. Another theoretical possibility is that Treg are preferentially preserved in HIV infection compared to other subsets. It has been shown, that Foxp3 represses retroviral transcription by targeting both NF- κ B and CREB pathways [274,275], which could contribute to the preservation of infected Treg harboring replication competent provirus.

Indeed, no statistically significant alteration in Treg absolute counts in viremic HIV patients could be demonstrated here, supporting a model of increased Treg frequencies leading to preserved Treg absolute counts. Admittedly, investigations in larger cohorts have described lower Treg absolute counts in viremic HIV patients [82,83,296–302]. While it is possible, that the statistical power in the present study was limited with 20 patients in the viremic patient group, it is informative to compare trends seen in the Treg population to those observed for Th17 cells in the same cohort.

The frequencies of Th17 cells within the CD4+ T cell population seemed to be stable in the peripheral blood of HIV infected patients in the present study, while their absolute counts were decreased in viremic patients, and restored by cART. In similar cohorts, both constant Th17 frequencies [264], and decreased Th17 absolute counts [94] have been described, even though Th17 abundance in the peripheral blood of HIV patients is far from clear, with many controversies in the relevant literature, as discussed in the subsequent section of this work.

Both Th17 and Treg absolute counts correlated strongly with CD4+ T cell counts in HIV patients, as reported by others for Treg [82,296,299] and Th17 [252]. The frequencies within the CD4+ T cell population of both these subsets correlated inversely with CD4+ T cell counts, but this correlation was much stronger for Treg. A strong inverse correlation of Treg frequencies with CD4+ T cell counts has been widely reported [266,296,297,300,301], whereas data for Th17 is limited, but a weak negative correlation with CD4+ T cell counts may exist [266]. Thus, in contrast to Treg, Th17 seemed to be less prone to a compensatory expansion in viremic patients with general CD4+ T cell depletion.

Furthermore, plasma viral loads correlated only with frequencies in the case of Treg (direct correlation), and only with absolute counts in the case of Th17 (inverse correlation). The correlation of Treg frequencies with plasma viral loads, reflecting the relative expansion of this subset in viremic patients is supported by many studies [82,83,296,297,301], although some found no relationship [82,83,300]. In contrast, limited data exists on the correlation of Th17 frequencies with plasma viral loads in HIV infection, but available studies also point towards a negative correlation with absolute Th17 counts [78,267]. This would seem to substantiate the hypothesis, that a compensatory mechanism related to plasma viremia acts to increase the frequency of Treg within the CD4+ T cell population, thereby keeping their absolute numbers relatively constant and, thus, independent of viral loads. On the other hand, Th17 have no similar compensatory mechanism, therefore Th17 frequencies are constant in HIV infection,

and absolute numbers are paralleling overall CD4+ T cell depletion, and hence are inversely correlated to plasma viral loads.

Here, a longitudinal analysis of Treg and Th17 populations in the peripheral blood of four HIV patients during eight years of cART was presented, starting with samples from before the initiation of therapy. Three of these patients had CD4+ T cell counts restored to healthy levels by cART, while one of them was a therapy non-responder, whose CD4+ T cell counts remained low, despite suppression of viral replication. No significant differences were detected in this small longitudinal cohort in the case of Treg, however, decreased Treg frequencies and increased Treg absolute counts after 1 year of cART had previously been shown in a larger longitudinal cohort [296].

In the longitudinal analysis, stable Th17 frequencies were detected. Moreover, Th17 absolute count restoration was observed in parallel to CD4+ T cell restoration in the three therapy responder patients, which complements the findings in the cross-sectional cohort. Very interestingly, there seemed to be an increase of Th17 frequencies in the therapy non-responder patient, causing a significant increase in Th17 numbers despite total CD4+ T cell numbers that remained low. Based on this finding, even though anecdotal in nature, one may argue, that Th17 are, in fact, capable of compensatory expansion, provided that viremia is suppressed.

In summary, a model is proposed, in which CD4+ T cell depletion is directly affecting both Th17 and Treg subsets, but the loss of Treg is compensated for by a relative expansion of this population in viremic HIV infection, while Th17 cells are less prone to such compensatory expansion when viral replication is ongoing. With suppression of viral replication, however, Th17 may also expand within the CD4+ T cell pool, causing a restoration of Th17 absolute numbers even if CD4+ T cell numbers remain low.

4.2. Definition of the Th17 subset based on surface marker co-expression versus ex vivo IL-17 production

The following discussion has been published previously [276].

Depletion of Th17 cells in the gut mucosa is a hallmark of HIV pathogenesis [78,80,94], but there is no consensus regarding Th17 frequencies in the peripheral blood in different stages of HIV disease [78–80,94,252,264–267]. Similar to the present findings, several other groups have reported constant frequencies of Th17 cells in the peripheral blood of viremic HIV patients based on IL-17 ICS [68,264] or the surface phenotype CXCR3-CCR6+CCR4+ [252] when compared to healthy donors. It is interesting to note that the authors reported a significantly lower frequency of Th17 cells in cART treated aviremic patients compared to healthy controls as well as viremic untreated patients in two of these cases [252,264], although the CD4 counts of the patients in these studies were lower and the average treatment times shorter than in the cohort investigated here. Several studies have shown a decreased frequency of Th17 cells in viremic HIV patients and some also found that their frequency was restored with long-term cART treatment to healthy levels [80,94,265,266,286]. Inconsistencies between different studies may partially be explained by the use of different phenotypes or gating strategies, or - since the vast majority of studies use IL-17 ICS as a preferred method - the differences of stimulation protocols.

Stem-cell properties [159] and a long half-life [158,160] have been recently described for these cells, and this could imply that Th17 cells may serve as major viral reservoir for HIV in patients undergoing cART [78,252,253,255,286]. This hypothesis warrants further investigation of Th17 cells in special HIV populations like *long-term nonprogressors* [305] and *elite controllers*. Live sorting protocols need to be validated for purposes of analyzing Th17 cells with regards to infection rates, gene expression profiles or T cell receptor repertoires in the context of HIV infection [252,268].

As part of this work, the determination of Th17 cells by two different Th17 phenotypes in both viremic and successfully treated HIV-infected subjects was evaluated.

The purpose was to investigate to what extent the surface marker phenotype CD4+, CD45RO+, CXCR3-, CCR6+, CCR4+, CD161+ correlates with Th17 cells that produce IL-17 without IFN γ after stimulation in ICS in this particular clinical context.

The main finding of the current study is an average sevenfold increase in the detected number of Th17 cells when defined by surface markers, as opposed to their definition by IL-17 production in healthy controls as well as in all stages of HIV infection. This finding is noteworthy, in so far as considerable plasticity of the Th17 subset has been described, i.e. a skewing of the Th17 population towards a Th1 phenotype [156,163,197,198], particularly in inflammatory conditions and at sites of inflammation [199,200]. Th1 cells that differentiate from Th17 cells secrete IFN γ instead of IL-17, and may lose [197] or maintain [156] CCR6 expression, while CD161 expression is maintained [156,200]. Of note, a lower MFI of CCR6, CCR4 or CD161 on CCR6+CXCR3-CCR4+CD161+ cells could not be detected in HIV infection, although a slight decrease in the frequency of CCR6+ cells within the CD4+CD45RO+ memory population was observed in viremic samples (not shown).

CXCR3 expression appears to be a good marker to distinguish cells committed to the Th1 lineage ("classic Th1 cells"); however, CXCR3 expression in "non-classic" (Th17-derived IL-17-IFN γ +) Th1 cells was reported to be lower [156]. Thus, Th17 derived Th1 cells may not express CXCR3 and stain as IL-17-IFN γ + by ICS. This phenomenon possibly contributes to the finding that the CCR6+CXCR3-CCR4+CD161+ phenotype considerably "overestimates" the number of *de facto* Th17 cells regardless of the HIV status.

Clearly, the present study has several limitations. First, no samples of patients with acute or recent HIV infection were included, while it is known that Th17 functionality and abundance are most gravely affected in this stage of the disease [79,80]. Furthermore, the study was performed on cryopreserved samples, however, no considerable differences of the frequencies of Th17 cells in fresh versus frozen samples were observed (data not shown), and the percentages obtained for the IL-17+IFN γ - as well as the CCR6+CXCR3-CCR4+CD161+ phenotype are comparable to relevant literature

[78,252]. Finally, the analysis did not include intracellular staining of the lineage specific master transcription factor ROR γ t, required for the expression of a Th17 phenotype [155]. While this would be the ideal gold standard to assess Th17 cells, intracellular staining with commercially available ROR γ t antibody (clone Q21-559, BD Biosciences) did not generate acceptable results in preliminary experiments of the present study (data not shown).

It is also possible that different combinations of surface markers (e.g. addition of IL-23R [77], CD26 [306]) might help to assess the frequency and number of Th17 cells in a more exact way. Another future direction, which requires the use of fresh PBMC samples, is the assessment and live sorting of Th17 cells by IL-17 secretion through an elaborate surface catch assay [200]. Alternatively, IL-17 surface staining [295] of freshly isolated PBMCs after stimulation with PMA and ionomycin is also possible, and may provide the closest appreciation of *de facto* Th17 cells by the exclusive use of a surface marker [295].

4.3. Assays for the quantification of proviral DNA loads and the replication competent reservoir in CD4+ T cell subsets

The latent reservoir of HIV in patients on long-term cART is primarily found in CD4+ T cells of the resting memory phenotype [210,251]. CD4+ T cells carrying latent provirus are rare in these patients, and may account for only 10^5 - 10^7 total HIV infected cells within a pool of approximately 10^{11} total CD4+ T cells in the body [307]. This rarity of latently HIV infected cells presents a technical challenge in studies aimed at quantifying the latent reservoir of HIV. Methods need to be both sensitive and specific to reliably quantify rare events representing latently HIV infected cells [38,280,308–310]. As any approach to cure HIV infection will ultimately necessitate elimination of the latent reservoir, the dynamic range of assays has to be robust enough to reproducibly detect small changes in reservoir size, in order evaluate the efficacy of experimental cure approaches [309,310]. Furthermore, an ideal assay to quantify the latent reservoir should

have the ability to discriminate between replication competent and defective forms of proviral HIV, as only the former has potential to cause rebound viremia [309,310].

The most widely used approaches for quantification of the HIV reservoir rely on PCR based quantification of the copy numbers of a chosen conserved sequence in the HIV genome, of which many have been published and are in use [277,311,312]. Provided that the number of input genomes is also quantified, HIV DNA copy numbers per cell can be measured by this method, and are usually reported as HIV copies/ 10^6 PBMC or CD4+ T cells. Real-time quantitative PCR allows for relative quantification of a DNA target using a standard curve generated by sequential dilutions of the template sequence in known copy numbers [313]. In contrast, Droplet Digital PCR provides a method for absolute quantification of the target sequence [314]. The DNA sample is distributed in thousands of oil droplets, and the droplets provide the vessel for the PCR reaction. Afterwards, droplets are analyzed for fluorescence, and discriminated as template negative and template positive droplets (end-point PCR). Based on this digital information, template copy numbers can be calculated assuming a random distribution of template in the droplets that is described by Poisson's law [314].

One of the aims in this study was to assess the potential of ddPCR to replace real-time PCR as a method of quantification of proviral DNA burden in PBMC and CD4+ T cells. It could be demonstrated, that these assays provide highly concordant results in quantification of template concentrations in clinical PBMC samples from HIV infected patients. Others have obtained similar results when comparing the two methods for quantification of HIV nucleic acids [290,315]. The average difference between the two assays is well within the range that is to be expected in a biological system, even though ddPCR quite consistently gave slightly lower concentrations as compared with real-time PCR. When quantifying HIV copies compared to input genome concentrations, slightly larger differences between the two methods were observed. In the case of ddPCR, a direct quantification of a cellular single copy gene, RPP30 [59] was performed, alongside HIV quantification within the same PCR reaction. In the case of

real-time PCR, total DNA concentrations quantified by microvolume spectrophotometry were applied to calculate genome counts, which is less accurate and reproducible, especially in the case of lower DNA concentrations [316,317]. While some real-time PCR platforms also allow for multiplex PCR reactions these usually require extensive calibration for each assay before use. Thus, the more straightforward duplexing capability of ddPCR provides an important advantage of this methodology over real-time PCR, especially in cases where sample quantities are limiting, which is often the case for clinical samples.

Importantly, increased sensitivity of ddPCR as compared with real-time PCR could not be confirmed in the present study. There are some clues in the literature that suggest an increased sensitivity of ddPCR with regards to small changes in copy numbers [315,318], however others also report no increased sensitivity in the case of HIV quantification when compared with real-time PCR [290,315]. Notably, one clinical sample was analyzed using both methods during the course of this study, where HIV copy numbers were below the limit of detection of real-time PCR, whereas ddPCR returned non-zero values. However, ddPCR has been reported to present an issue of false positives, specifically single template-positive droplets have been observed [290,315]. Therefore, PBMC samples of four HIV-uninfected donors were assessed by ddPCR for HIV-LTR, and up to two false positive droplets were detected in some cases (not shown). Analysis of the false positive rate using all negative controls across all our measurements determined three positive droplets as call threshold for sample positivity in the HIV-LTR assay (confidence level 99%).

The absolute quantification in the case of ddPCR is probably responsible for its superior reproducibility in time, and between different operators when compared with qPCR [318,319].

In summary, ddPCR represents an attractive alternative for real-time PCR when quantifying HIV copy numbers in clinical samples. While increased sensitivity of this method is not evident, especially because of reported issues regarding false positives,

the absolute quantification and the option to easily co-quantify cellular genomes based on a genomic single-copy gene offered by ddPCR are significant advantages over real-time PCR.

Even though PCR based quantification of selected HIV DNA sequences in cellular DNA samples is technically straightforward, it does not allow for discrimination of proviral DNA coding for replication competent versus replication defective virus. Viral outgrowth assays (VOA) relying on limiting serial dilution of purified resting CD4⁺ T cells, followed by activation with PHA, a strong T cell mitogen in cell culture have been in use for 20 years to estimate the fraction of CD4⁺ T cells harboring replication competent provirus [210]. This method is still considered the “gold standard” for the assessment of the replication competent reservoir by some experts [309].

In this study, ddPCR was successfully implemented as a single platform for proviral DNA quantification, as well as detection of viral outgrowth from cell culture supernatants. Other studies quantifying HIV reservoir in CD4⁺ T cell subsets have used quantitative PCR for proviral DNA quantification, while viral outgrowth was detected by enzyme-linked immunosorbent assay for detection of the p24 HIV protein [238,254,271], or alternatively, firefly luciferase activity in the TZM-bl cell line [253,280]. These approaches have the disadvantage of necessitating the establishment of multiple methodologies to comprehensively investigate viral reservoirs. Furthermore, the RNA based detection of viral outgrowth used in the present study may be more sensitive and therefore requires shorter culture times [281].

VOA consistently reports range of one-in-a-million CD4⁺ T cells carrying replication competent provirus [210,280,281], which is on average at least 300 fold lower, than the frequencies reported for integrated HIV DNA carrying CD4⁺ T cells [37,38]. This is in itself not surprising, given the high mutation rate of HIV, and the fact that CD4⁺ T cells harboring replication competent virus are expected to have a shorter half-life, because of eventual viral reactivation and lysis or clearance by the immune system.

Based on these considerations, PCR based quantification of proviral DNA is bound to considerably overestimate the clinically relevant portion of the HIV reservoir. It is debated, whether PCR based approaches may still be meaningful, because data exists to support correlation of VOA results with PCR based measurements [280], whereas others have shown no correlation [38].

On the other hand, it has been demonstrated, that VOA might underestimate the replication competent reservoir [37], because intact proviral DNA sequences have been recovered from cell culture wells without detectable viral outgrowth, and reconstruction of the corresponding viruses demonstrated growth kinetics that were comparable to viruses that were successfully induced in the VOA [37]. Recently, a novel cell culture assay named *tat/rev* Induced Limiting Dilution Assay (“TILDA”), has been proposed to replace VOA [308]. Briefly, TILDA relies on detection of multiply spliced viral *tat/rev* RNA product as a surrogate marker for replication competent virus. While this approach considerably shortens the assay time, TILDA failed to correlate with either VOA or PCR based quantification in CD4+ T cells [308]. Furthermore, *tat/rev* multiply spliced RNA seems to be detectable in cells with defective proviruses, where viral particle production does not take place [320].

To conclude, current state of the art requires quantification of both total proviral DNA copy number, as well as the replication competent virus to provide a complete picture of the reservoir size. HIV reservoir quantification is an area of intensive research, based on its pivotal importance in evaluating the effectiveness of any HIV eradication study. The near future will probably see the publication of new approaches, and Droplet Digital PCR based quantification of proviral DNA and replication competent virus is likely to have a role.

4.4. Detailed assessment of the HIV-1 reservoir in different CD4+ T cell subsets

The main goal of this work was to assess the role of Th17 and Treg cells as possible reservoirs of replication competent virus. As described above, the first half of this study demonstrated that live-sorting Th17 cells based on combined expression of published surface markers is unlikely to yield a phenotypically pure population. The possibility to sort Th17 cells based on surface expression of IL-17 exists [295]; this method, however, yielded too few cells in the peripheral blood to allow for relevant measurement of HIV reservoirs. Thus, the remainder of the work focused on investigating the HIV reservoir in Treg as compared with Tcm and Tem.

In the present study, the role of Treg as a reservoir site of proviral HIV, including replication competent virus in chronic infection was investigated. No differences were observed between Treg, Tcm and Tem in terms of proviral DNA load. With regards to HIV copy numbers in Tcm and Tem, the presented results showed values that are comparable to previous findings [251,253,284], considering the different approaches in quantification. Namely, the authors of these works use real-time *Alu-gag* PCR [312], a nested PCR based method that is incompatible with ddPCR, designed to selectively quantify integrated HIV DNA. Admittedly, the present ddPCR based approach might theoretically amplify extrachromosomal forms of HIV DNA (such as 2-LTR circles) [315,321], that do not constitute a reservoir of HIV. These forms of HIV DNA are, however, extremely rare in patients without detectable viral replication [38], and their effect as contaminants in our measurements are likely to be negligible. Furthermore, it has to be noted, that the definition of the Tem subset applied in this study does not discriminate the CD27+ transitional memory (Ttm) subset described in these works [251,253,284].

In case of Treg, the duplex ddPCR assay determined proviral DNA loads that are considerably higher than reported previously by qPCR based measurements in cohorts with similar characteristics [254,271,272]. It is worth noting, that the approach of genomic input estimation in these studies is different, since the authors either apply

parallel quantification of a genomic single copy gene ([271] and the present study), or provide an estimate based on counts of sorted cells [272], or a limiting dilution of genome equivalents of DNA [254]. In light of the presented results, Treg do not appear to be a preferential reservoir site for HIV when compared to other memory subsets, instead of all CD4+ non-Treg T cells, as investigated previously [254,271,272]. Similar infection rates of Treg compared to memory CD4+ T cells have also been reported in patients with ongoing virus replication [273].

In this study a successful quantification of Treg harboring replication-competent HIV provirus in two HIV infected donors on long-term suppressive cART has been presented. In these two individuals, the replication competent reservoir in Treg was similar to Tcm and Tem.

In three of the five donors investigated, viral outgrowth could not be detected from Treg. It has to be recognized, that the rarity of replication competent virus in HIV patients on long time suppressive cART may necessitate a larger number of cells to rule out stochastic effects. Even though samples from a limited number of patients were analyzed in this laborious and multistep *in vitro* assay, it cannot be excluded, that differences exist in subtype specific permissiveness to HIV reactivation between the CD4+ T cell subsets investigated in this study. A different transcriptional regulation of viral reactivation in case of Treg has also been proposed by Chase and colleagues, who have not been able to detect viral outgrowth from sorted Treg of patients of suppressive antiretroviral therapy [272]. As already discussed, overexpression of Foxp3 has been shown to repress HIV transcription [274,275]. Sequencing of the proviral genomes in Treg compared to other CD4+ T cells subsets might be an interesting aim to pursue, and may shed some light on differences of HIV reactivation in various CD4+ T cell populations [37,251,253].

In summary, the question whether peripheral regulatory T cells represent a significant reservoir for HIV has been reassessed in this study. Since higher frequencies of cells carrying proviral HIV DNA in Treg could not be observed when compared to Tcm

or Tem, the conclusion is that Treg have the preponderance of proviral HIV that is to be expected based on their mostly memory phenotype. The replication competent viral reservoir in Treg was successfully quantified in a limited number of cases. However, viral outgrowth in Treg was somewhat limited when compared to the other investigated subsets, in spite of similar proviral DNA burden and complete cellular activation in cell culture. Further studies are needed to investigate whether a different permissiveness to HIV reactivation might exist in the Treg subset when compared to memory cells of non-Treg origin.

5. Summary

This work has been carried out in order to assess the possible role of CD4+ T cell subpopulations Th17 and Treg, as reservoirs of HIV in patients on long-term cART. Furthermore, the potential use of Droplet Digital PCR was demonstrated in quantifying proviral DNA using relatively small amounts of blood samples in these patients with very low viral loads. In parallel, the replication competent HIV reservoir was assessed in these clinical samples by use of a quantitative viral outgrowth assay (qVOA).

At the same time, frequencies of Th17 and Treg cells in peripheral blood were investigated in a cohort of HIV patients. In agreement with earlier findings, the absolute counts of Th17 were shown here to be decreased in viremic HIV patients, while their numbers were restored to near healthy levels by long-term antiretroviral therapy.

To determine the frequencies of Th17 cells, a surface marker based identification of this subset was performed in parallel with a direct identification of IL-17 producing cells after *in vitro* stimulation with phytohaemagglutinin and ionomycin (intracellular cytokine staining, ICS). In particular, it could be demonstrated that the identification of these cells based solely on published surface markers considerably overestimates the number of IL-17 producing CD4+ T cells.

To determine the size of the reservoir in Th17 cells, IL-17 expressing cells of HIV patients have been isolated based on surface expression of this cytokine. However, the amount of cells obtained using this method in peripheral blood samples was insufficient to quantify the HIV reservoir by ddPCR and qVOA.

The CD4+ Treg subpopulation was analyzed in the context of viremic and treated HIV infection, and previous findings could be confirmed, showing that Treg frequencies are relatively increased in the case of low CD4+ T cell counts and in viremic patients.

A study of the HIV reservoir in Treg has been presented, in a cohort of 10 chronically HIV infected patients on cART. No differences between proviral DNA loads and

replication competent viral reservoir between Treg, central and effector memory (Tcm and Tem) cells could be demonstrated. Thus, this study presented evidence that may require the re-evaluation of the role of Treg as a privileged cellular reservoir of HIV, demonstrating that their proviral HIV burden is similar to that of memory subsets.

5. Zusammenfassung

Das Ziel der vorliegenden Arbeit war es, die Rolle von Th17 und regulatorischen T Zellen (Treg) als mögliches Reservoir des HI-Virus (HIV) bei langjährig antiviral behandelten HIV Patienten zu erfassen. Darüber hinaus wurde die Eignung der *Droplet Digital PCR* Technologie, eine neue Methode für die Quantifizierung proviraler DNA Sequenzen bei diesen Patienten mit sehr niedriger Viruslast und wenig Ausgangsmaterial demonstriert. Parallel wurde das replikationskompetente HIV Reservoir in diesen klinischen Blutproben mittels *quantitative viral outgrowth assay* (qVOA) erfasst.

Zeitgleich wurden die Frequenzen von CD4+ Th17 Zellen und CD4+ Treg Zellen in einer ausgewählten HIV Kohorte analysiert. Übereinstimmend mit der Literatur konnte gezeigt werden, dass die absolute Anzahl der Th17 Zellen im peripheren Blut in der unbehandelten HIV Infektion sinkt. In langzeitbehandelten Patienten konnte jedoch eine langsame Restoration der absoluten Anzahl von Th17 Zellen beobachtet werden.

Zur Erfassung der Frequenz der Th17 Zellen wurden im Rahmen dieser Arbeit zwei etablierte Methoden eingesetzt. Einerseits wurden die Zellen durch eine Reihe von Oberflächenmarkern identifiziert, andererseits wurde diese Subpopulation parallel auch direkt anhand der *in vitro* IL-17 Produktion nach Stimulation mit Phytohaemagglutinin und Ionomycin im Intrazellulären Zytokin Assay (ICS) untersucht. Durch den Vergleich der beiden Methoden konnte demonstriert werden, dass der Nachweis von Th17 Zellen ausschließlich mit Hilfe von Oberflächenmarkern die Zahl der tatsächlich IL-17-produzierenden Zellen stark überinterpretiert.

Zur Erfassung des HIV Reservoirs in Th17 Zellen wurden IL-17 produzierende Zellen spezifisch anhand ihrer IL-17 Produktion isoliert. Allerdings konnte aus peripheren Blutproben nicht genügend Material zur Ausführung weitergehender Experimente zur Quantifizierung des Reservoirs (qVOA, ddPCR) gewonnen werden.

Die Analyse der Treg Zellen in der Kohorte ergab eine Zunahme der relativen Fre-

quenz der Treg Zellen in den CD4+ T Zellen von Patienten mit nachweisbarer Viruslast und einer geringen Anzahl an CD4+ T Zellen.

Zur Beantwortung der Frage, ob regulatorische Zellen ein HIV Reservoir darstellen, wurden Treg Zellen aus klinischem Material von zehn HIV infizierten Patienten isoliert. Der Gehalt proviraler HIV DNA wurde mittels ddPCR bestimmt und der Anteil replikationskompetenter Viren mittels qVOA untersucht. Es konnten keine signifikanten Unterschiede an proviralen DNA Mengen oder replikationskompetenten Viren zwischen verschiedenen CD4+ Subpopulationen Treg, Zentrale und Effektor-Gedächtniszellen (Tcm und Tem) nachgewiesen werden. Diese Untersuchungen geben zudem Hinweis darauf, dass die Rolle der Treg Zellen als bevorzugtes Reservoir für das HI-Virus im Vergleich zu anderen CD4+ Gedächtniszellen nicht eindeutig belegbar ist.

6. Abbreviations used in this work

| | |
|--------------------------------------|---|
| AIDS | acquired immune deficiency syndrome |
| Alu | genomic transposable element (characterized by <i>Arthrobacter luteus</i> restriction endonuclease) |
| ANOVA | analysis of variance |
| ANRS | Agence National de Recherche sur le SIDA (National Agency for AIDS reserach) |
| APOBEC3G | apolipoprotein B mRNA-editing enzyme catalytic polypeptide 3G |
| Bcl6 | B cell lymphoma 6 |
| BLIMP-1 | B lymphocyte induced maturation protein 1 |
| CA | capsid |
| cART | combination anti-retroviral therapy |
| CCL(28, etc) | C-C chemokine ligand |
| CCR(5,6,7 etc) | C-C chemokine receptor |
| CD(3,4, etc) | cluster of differentiation |
| CNS | central nervous system |
| CRF | circulating recombinant form |
| cSMAC | central supramolecular activation complex |
| CTLA-4 | cytotoxic T lymphocyte associated protein 4 |
| CTS | central termination sequence |
| CXCR(4, etc) | C-X-C chemokine receptor |
| DC | dendritic cell |
| ddPCR | Droplet Digital PCR |
| DNA | deoxyribonucleic acid |
| ESCRT | endosomal sorting complexes required for trafficking |
| FACS | fluorescence activated cell sorting |
| FOXP3 | forkhead box protein 3 |
| HEXIM1 | hexamethylenebisacetamide-induced protein 1 |
| hi (ie. T-bethi) | high expression |
| HIV | human immunodeficiency virus |
| HLA(-A,B,C,DR...) | human leukocyte antigen |
| ICOS(-L) | inducible costimulator receptor(-ligand) |
| ICS | intracellular cytokine staining |
| IFN(α , β , γ) | interferon |
| IN | integrase |
| iTreg | induced regulatory T cell |
| iTreg | induced Treg |
| IUPM | infectious units per million |
| Jak(1, 3, etc) | Janus kinase |
| LAG-3 | lymphocyte activation gene 3 |
| LCA | leukocyte common antigen |

| | |
|------------------|---|
| LCMV | lymphocytic choriomeningitis virus |
| lo (ie. CD127lo) | low expression |
| LTR | long terminal repeat |
| MA | matrix |
| MAIT | mucosa-associated invariant T cell |
| MHC | major histocompatibility complex |
| MSM | men who have sex with men |
| NC | nucleocapsid |
| NFAT | nuclear factor of activated T cells |
| NF- κ B | nuclear factor κ B |
| NK | natural killer cell |
| NKT | natural killer T cell |
| NTC | no template control |
| nTh17 | natural Th17 cell |
| nTreg | natural regulatory T cell |
| ORF | open reading frame |
| p:MHC | antigen peptide presented on major histocompatibility complex |
| PBMC | peripheral blood mononuclear cell |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PD-1 | programmed death 1 |
| PHA | phytohaemagglutinin |
| PIC | pre-integration complex |
| PPAR γ | peroxisome proliferator-activated receptor γ |
| PPT | polypurine tract |
| PR | protease |
| pTEFb | positive transcription elongation factor b |
| qPCR | quantitative real-time PCR |
| qVOA | quantitative viral outgrowth assay |
| Rev | regulator of expression of virion proteins |
| RNA | ribonucleic acid |
| ROR γ t | retinoid orphan receptor γ t |
| RPMI | Roswell Park Memorial Institute 1640 medium |
| RPP30 | Ribonuclease P protein subunit p30 |
| RT | reverse transcriptase |
| RTC | reverse transcription complex |
| SAMHD1 | SAM domain and HD domain containing protein 1 |
| SEM | standard error of measurement |
| SIV | simian immunodeficiency virus |
| SP1 | specificity protein 1 |
| STAT(4, 5, etc) | signal transducer and activator of transcription |

| | |
|-----------------------|---|
| STD | sexually transmitted disease |
| SU | surface |
| T-bet | T-box expressed in T cells |
| TAR | trans-activation region |
| Tat | trans-activator of transcription |
| Tcm | central memory T cell |
| TCR | T cell receptor |
| Teff | effector T cell |
| Tem | effector memory T cell |
| Tfh | T follicular helper cell |
| TGF β | transforming growth factor β |
| Th(1, 2, 17, 22, etc) | T helper cell |
| TILDA | Tat/rev Induced Limiting Dilution Assay |
| TM | transmembrane |
| Tn | naive T cell |
| Treg | regulatory T cell |
| TRIM5 α | tripartite motif protein 5a |
| Tscm | stem cell memory T cell |
| Ttd | terminally differentiated T cell (also Tte) |
| Tte | terminally differentiated T cell (also Ttd) |
| Ttm | transitional memory T cell |
| VDJ | Variability Diversity Joining |

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9.6. Methodenkenntnisse

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10. Publikationen

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Dunay GA, Tóth I, Eberhard JM, Degen O, Tolosa E, van Lunzen J, Hauber J, zur Wiesch JS. Parallel assessment of Th17 cell frequencies by surface marker co-expression versus ex vivo IL-17 production in HIV-1 infection. *Cytom. Part B Clin. Cytom.* *In press.*

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Dunay GA, Paragi P, Sára L, Ács N, Balázs B, Ágoston V, Répás C, Ivanics T, Miklós Z. Depressed calcium cycling contributes to lower ischemia tolerance in hearts of estrogen-deficient rats. *Menopause* 2015;22:773–782.

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Miklos Zs, Dunay G, Kemecei P, Tokes A, Ivanics T. Activation of renin angiotensin system contributes to the impairment of intracellular calcium handling via angiotensin receptor 1 signaling pathways in the diabetic heart. *Acta Physiologica* (2011) 202 (S 684) pp 58.

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Konferenzbeiträge

Dunay GA, Solomatina A, Hufner AD, Degen O, Tolosa E, Hauber J, Schulze zur Wiesch J. The potential of Droplet Digital PCR technology for the quantification of HIV-1 proviral DNA and replication competent virus in different CD4⁺ subsets. 26th Annual Meeting of the Society for Virology, 6-9 April 2016, Münster, Deutschland.

Kobbe G, Kaiser R, Knops E, Luebke N, Dunay GA, Fischer J, Huettig, F, Haas R, Haeussinger D, Jensen BO. Treatment of HIV and AML by Allogeneic CCR5-d32 Blood Stem-Cell Transplantation. Conference on Retroviruses and Opportunistic Infections (CROI), Boston MA, 22-25 Februar, 2016.

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11. Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

A handwritten signature in black ink, reading "Dunay Gábor". The signature is written in a cursive, flowing style.

Unterschrift: Dr.med. Gábor A. Dunay