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Analysis of viral reservoirs in chronically SIV-infected sooty mangabeys treated with combination antiretroviral therapy (cART)

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Abstract

ABSTRACT

***Background**: Human Immunodeficiency Virus (HIV), the causative pathogen of Acquired Immunodeficiency Syndrome (AIDS), continues to be a major global public health issue. Although, potent antiretroviral therapy (ART) has dramatically reduced the morbidity and mortality in HIV-infected individuals, one of the most consistent obstacles to HIV eradication is the presence of stable viral reservoirs of latently infected cells. The aim of the present study is to understand the mechanisms of the complex virus-host interactions that lead to persistent infection, and to achieve the ideal combination of therapies to eliminate HIV virus in an non human primate model, Sooty Mangabeys (SMs), that preserves CD4+ T cells homeostasis, avoiding the AIDS progression which occurs in Rhesus Macaque (RM) and humans.*

***Materials and Methods**: Twelve experimental chronically SIV-infected SMs, not homozygous for CCR5-null alleles and with viral load of 10^3-10^5 viral RNA copies/ml, were treated with combination antiretroviral therapy (cART) regimen consisting of Tenofovir, Emtricitabine, Raltegravir and Darunavir. The selected SMs were divided in four-treatment interruption groups receiving cART up to 2, 6, 9 and 12 months. Plasma viral loads were detected by RT PCR, while CD4+ T subsets dynamics and their proliferation and activation status were analyzed by flow cytometry in different anatomical compartments and prior to the treatment initiation and during the cART in all SMs. cART-induced variations in CD4+ T cell subsets susceptibility to SIV infection were evaluated through FACS procedure and cell-associated SIV-DNA assay.*

***Results**: No severe cART-related side effects, in terms of body weight and renal function indices were observed. Eleven out of twelve treated animals experienced a 2-3 log decline of plasma viremia at the earliest time points, below the level of detection. Although, analysis of total circulating $CD4^+$ population showed minor changes in terms of frequency and absolute number, a significant recovery of CD4+ T cells in the mucosal compartment was observed. Interestingly, the study of CD4+ T cells subsets in the blood, highlighted a rapid and marked reduction of frequency and absolute count of effector memory (EM) and an expansion of central memory (CM) and memory stem cells (SCM) at early time point after ART initiation in SMs. cART resulted to be efficient in reducing immune activation levels on CD8+ T cells both in blood and mucosal compartments. In response to cART, a generalized reduction in SIV-infected CD4+ T cell subsets was observed and specifically in the fraction that represented the main virus source, i.e, EM and transitional memory T cells (TM). Analysis of cART interruption revealed that although, the group receiving cART for 2 months, experienced a rapid viral rebound after therapy interruption, interestingly, a control in viremia was observed after 6 months of cART in the treated animals.*

***Conclusion**: The four-drugs regimen proved to be safe, well tolerated with no discernible side effects and effective in suppressing viral replication in treated SMs. cART induced immunological changes and specifically a significant reduction of immune activation in both blood and mucosal compartments. A redistribution of CD4+ T cell subsets and a generalized decrease of CD4+ cellular subsets harboring virus in the early phases of treatment is a favorable scenario at*

promoting further reduction of SIV reservoirs or their clearance for prolonged cART periods (9-12 months).

SOMMARIO

***Introduzione**: Il virus dell'immunodeficienza umana (HIV), agente patogeno della sindrome da immunodeficienza acquisita (AIDS), continua ad essere uno dei maggiori problemi globali di salute pubblica. Sebbene, la potente terapia antiretrovirale (ART) abbia drasticamente ridotto la morbidità e la mortalità nei soggetti con infezione da HIV, uno degli ostacoli più consistenti per l'eradicazione del virus, è la presenza di stabili serbatoi virali in cellule latentemente infette. Lo scopo del presente studio è quello di comprendere i meccanismi alla base delle complesse interazioni tra virus-ospite che contribuiscono alla persistenza dell'infezione e di ottenere un'ottimale combinazione di terapie atte ad eliminare l'HIV in un modello di primate (cercocebo moro – SMs). Questo modello preserva i linfociti CD4, evitando la progressione verso l'AIDS a differenza del Macaco Rhesus (RM) e degli uomini.*

***Materiali e Metodi:** Dodici SMs infettati sperimentalmente con SIV in fase cronica, non omozigoti per la mutazione del gene CCR5 e con carica virale di 10^3-10^5 copie di RNA/ml, sono stati trattati con una combinazione di farmaci antiretrovirali (cART) composta da Tenofovir, Emtricitabine, Raltegravir e Darunavir. I cercocebi mori sono stati divisi in quattro gruppi a cui è stato effettuato il trattamento rispettivamente per 2, 6, 9 e 12 mesi. La carica virale è stata rilevata su campioni di plasma mediante RT-PCR, mentre le dinamiche dei sottotipi cellulari dei linfociti CD4, il loro stato di proliferazione ed attivazione, sono stati analizzati mediante citofluorimetria a flusso in diversi compartimenti anatomici, prima e durante la terapia. Variazioni nella suscettibilità all'infezione da SIV in seguito a somministrazione della cART, sono state valutate nei diversi sottotipi cellulari dei linfociti CD4, mediante procedura di FACS e saggio di quantificazione del DNA virale totale.*

***Risultati:** Non sono stati riscontrati gravi effetti collaterali dovuti alla terapia, in termini di peso corporeo e indici di funzionalità renale. Undici dei dodici animali trattati hanno mostrato inizialemente una riduzione di 2-3 log della viremia plasmatica, inferiore ai limiti di detezione. Sebbene l'analisi dei linfociti CD4 circolanti abbia mostrato minori variazioni in termini di frequenza e conta, un ripristino significativo dei linfociti CD4 è stato osservato a livello delle mucose. E' interessante notare che l'analisi dei sottotipi cellulari dei linfociti CD4 nel sangue, ha evidenziato una rapida e significativa riduzione delle cellule effetrici della memoria (EM) ed un incremento delle cellule della memoria centrale (CM) e delle cellule T staminali di memoria (SCM) in tempi precoci in seguito alla somministrazione della cART. Nel sangue e nelle mucose, la cART si è rivelata efficace nella riduzione dei livelli di attivazione dei linfociti CD8. Per effetto della terapia, è stata inoltre osservata una riduzione generalizzata dell'infezione da SIV in tutti i sottotipi dei linfociti CD4 ed in particolare, nella frazione cellulare maggiormente coinvolta, ossia le EM. Al momento dell'interruzione della terapia, il gruppo trattato per due mesi ha evidenziato una rapida replicazione virale, mentre nel gruppo trattato per 6 mesi la viremia rimane controllata.*

***Conclusioni:** La terapia ha dimostrato di essere sicura, ben tollerata, senza particolari effetti collaterali, ed efficace nella soppressione della replicazione virale negli animali trattati. La cART ha indotto alcune variazioni immunologiche tra cui una riduzione dell'immunoattivazione nel compartimento sistemico e mucosale. Giá*

a partire dalle fasi iniziali. la ridistribuzione dei sottotipi cellulari dei linfociti CD4 ed una generalizzata riduzione nel contenuto di DNA virale in risposta al trattamento, delineano uno scenario favorevole per un ulteriore decremento delle dimensioni dei serbatoi virali o per la loro completa eliminazione in seguito a periodi prolungati di terapia (9-12 mesi).

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LIST OF ABBREVIATIONS

AGMs: African Green Monkey

AIDS: Acquired Immune Deficiency Syndrome

APOBEC: APOlipoprotein B mRNA-editing Enzyme Catalytic polypeptide-like editing complex 3G

ART: Anti-Retroviral Therapy

CA: Capsid

cART: Combination Anti-Retroviral Therapy

CD4: Lymphocytes T CD4 positive

CD8: Lymphocytes T CD8 positive

CTL: Cytotoxic T Lymphocyte

DC: Dendritic Cells

DMSO: DiMethyl SulfOxide

DN: Double Negative

DNA: DeoxyriboNucleic Acid

DRV: Darunavir

ENV: Envelope

FACS: Fluorescence-Activated Cell Sorting

FBS: Fetal Bovine Serum

FDA: Food and Drug Administration

FDC: Follicular Dendritic Cells

FTC: Beta-2',3'-dideoxy-3'-thia-5-FluoroCyTidine -Emtricitabine

GALT: Gut-Associated Lymphoid Tissue

HAART: Highly Active Anti-Retroviral Therapy

HIV: Human Immunodeficiency Virus

HIV-1: Human Immunodeficiency Virus type 1

HIV-2: Human Immunodeficiency Virus type 2

IL: InterLeukin

INIs: INtegrase Inhibitors

LN: Lymph Node

LNTPs: Long-Term Non Progressors

LPS: LipoPolySaccharide

LTR: Long Terminal Repeat

MA: Matrix

MALT: Mucose-Associated Lymphoid Tissue

MNDs: Mandrills

NHP: Non Human Primate

NK: Natural Killer

NNRTIs: Non-Nucleoside Reverse-Transcriptase Inhibitors

NRTIs: Nucleoside Reverse-Transcriptase Inhibitors

PB: Peripheral Blood

PBMCs: Peripheral Blood Mononuclear Cells

PIs: Protease Inhibitors

PMPA: 2-PhosphoMomethoxyPropyl Adenine - Tenofovir

RB: Rectal Biopsy

RLT: Raltegravir

RMs: Rhesus Macaques

RNA: RiboNucleic Acid

RT: Reverse Transcriptase

SHIV: Simian/Human Immunodeficiency Virus

SIV: Simian Immunodeficiency Virus

SIVagm: Simian Immunodeficiency Virus African Green Monkey subtype

SIVmac: Simian Immunodeficiency Virus Rhesus MACaques

SIVsmm: Simian Immunodeficiency Virus Sooty Mangabeys subtype

SMs: Sooty Mangabeys

TCM: Central Memory T cells

TEM: Effector Memory T cells

Th17: T helper 17 cells

T reg: T regulatory cells

TSCM: T Memory Stem Cells

TTM: Transitional Memory T cells

WT: WildType

LIST OF FIGURES

Introduction

1. INTRODUCTION

1.1 Acquired Immunodeficiency Syndrome (AIDS) history and its Epidemiology

Despite more than 30 years of active research, the Human Immunodeficiency Virus (HIV), the causative pathogen of Acquired Immunodeficiency Syndrome (AIDS), continues to be a major global public health issue. Approximately 35 million people living with HIV were estimated at the end of 2013. This increased number is the result of a major accessibility to antiretroviral therapy with respect to previous years. However, the rate of new infections is still high, with 2.1 million people becoming newly infected with HIV globally. The number of AIDS deaths has increased with respect to 2012 (1.6 million), having claimed more than 39 million lives so far in 2013 [1], confirming that AIDS is an ongoing challenge.

Moreover, the difficulty of diagnosis of AIDS symptoms, that appear to be of nonspecific nature, easily confused with a variety of other illnesses, does not help the health care providers and may explain why this infection has not been discovered until 1981. In that year, a group of young gay men in New York, San Francisco and Los Angeles were diagnosed with symptoms not usually observed in individuals with a healthy immune status [2, 3, 4]. In particular, a very aggressive form of Kaposi's sarcoma or *Pneumocystis carinii pneumonia,* lung infection, was observed in these individuals [4]. Due to its first occurrences in male homosexual community, the disease was also temporarily called gay compromise syndrome or gay-related immune deficiency (GRID) [5]. However, after determining that AIDS was not isolated to the gay groups [6, 7], it was realized that the term GRID was misleading, so, in 1982, it was attributing a new name, AIDS, by CDC (Centers for Disease Control and Prevention). Soon, the reported new

cases soared, with more than hundreds people, above all among the younger. In the individuals hit by AIDS, an extremely severe clinical course and high number of deaths was observed. It made the world understand that a new disease associated with the breakdown of the body's immune system was appeared. Finally, in 1983, AIDS pathogen was isolated from lymphoid ganglions of an infected patient by Luc Montagnier and Françoise Barré-Sinoussi at the Institute Pasteur in Paris, attributing it the name lymphadenopathy-associated virus (LAV) [8]. Independently, at the same time, Robert Gallo's research group declared to have found the retrovirus that may have been infecting AIDS patients, calling it HTLV-III [9]. In May 1986, the International Committee on Taxonomy of Viruses coined a new name, HIV (Human Immunodeficiency Virus) [10].

Despite the identification of AIDS etiologic agent in 80s and the achieved goals in HIV research fields with extraordinary changes in the AIDS landscape, it's well established that some obstacles in HIV elimination still remain. First of all, the presence of epidemic burden that varies considerably between regions and countries over the world. Sub-Saharan Africa is one of the most affected regions, accounting for 24.7 million people living with HIV in 2013 and for almost 70% of the global total of new HIV infections [11]. This is likely the result of many factors, including education, morals, religion, virus transmission routes, different availability of antiretroviral drugs, stigmatization of HIV infection and higher rapes frequency [12]. Moreover, this high prevalence in Sub-Saharan Africa could probably also due to the fact that this is the region where HIV was transmitted first in humans through multiple infections from simian immunodeficiency virus (SIV)-infected nonhuman primates [13].

In other countries, such as Asia, almost 4.8 million people are living with HIV, although the regional prevalence of HIV infection is about one-seventeenth that in sub-Saharan Africa. In contrast in Western and Central

Europe HIV prevalence rates are about 0.1% [14]. Progress has been dramatic in stopping new HIV infections and, above all, among children. In 2013, 240 000 children were newly infected with HIV, 58% lower than 2002. Despite the positive achieved successes to limit AIDS pandemic, there is still a long way to go. According to the World Health Report in 2008 [14] for every two people starting to take antiretroviral therapy, another five become newly infected. The main focus in low-income countries is not to treat patients but to prevent the onset of new infections with the design of HIV vaccines. Until then, clinical diagnostic tools to select appropriate antiretroviral therapies will become even more important and serve as a valuable factor in the fight against HIV.

1.1.1 Clinical course of HIV Infection

HIV can be transmitted through several routes: by sharing injecting equipment, by receiving blood transfusions or other blood related products from an infected person (in particular among intravenous drug abusers), from an HIV-positive mother to the baby (during pregnancy, childbirth and breast feeding) and mainly spread by risky homo and bisexual activities. In these ways, the contact of mucosal surfaces or damaged tissues with the body fluids of an HIV-infected person make the transmission possibly occurs.

The clinical course of HIV infection generally includes three phases: primary infection, clinical latency and AIDS-defining illness [15] (Figure 1). Within the first week of exposure, at the sites of virus entry, HIV is transported to local lymph nodes by Langerhans cells (LC) or dendritic cells (DCs). From those sites, HIV can spread and reach the other body districts, particularly gut-associated lymphoid tissue (GALT), the principal site of HIV infection and a reservoir of the virus that supports its persistence even during long-term viremia suppression in presence of antiretroviral therapy [16, 17]. At around 3-12 weeks after infection, virus appears in the blood

with burst of viremia (up to 10^7 HIV RNA copies per ml of plasma) that favors HIV dissemination throughout the body and the infection of helper T-cells, macrophages and DCs in peripheral lymphoid tissues. This phase commonly named *acute phase* of HIV infection, is therefore accompanied by a rapid depletion of the memory CD4+CCR5+ T cells, resulting in a variety of nonspecific signs and symptoms typical of many viral diseases.

By 16 weeks after infection, the symptoms of acute viremia passed, but virus persists in the plasma. Resolution of the clinical syndrome, typical of acute phase and drop in viremia generally occur into a stage called "*clinical latency*" phase that is associated with the emergence of HIV-specific host immune responses. Indeed, the immune system partially controls the infection and viral production, as reflected by a drop in viremia. The chronic and clinically asymptomatic latent phase of HIV infection can last for a period of 8-10 years and the virus continues to reproduce at very low levels. Lymph nodes (LNs) and the spleen are sites of continuous HIV replication and cell destruction. Although the majority of peripheral blood T-cells does not harbor the virus, destruction of CD4+T-cells within lymphoid tissues steadily progresses during the latent period, and the number of circulating blood CD4+ T-cells steadily declines.

The progression to AIDS-defining illness, the last stage of the disease, is characterized by CD4+ T cell counts below 200 cells per mm^3. In this condition, HIV individuals become susceptible to other infections and the immune responses against new pathogens may stimulate HIV production and accelerate the destruction of lymphoid tissues. Viremia may dramatically climb, sustained also by the residual ongoing viral replication in the latent reservoirs. Generally the impossibility of new T cells regeneration and the presence of opportunistic protozoal, bacterial, viral and fungal infections or malignancies drive HIV-infected patients to the death after some years of disease [15,16].

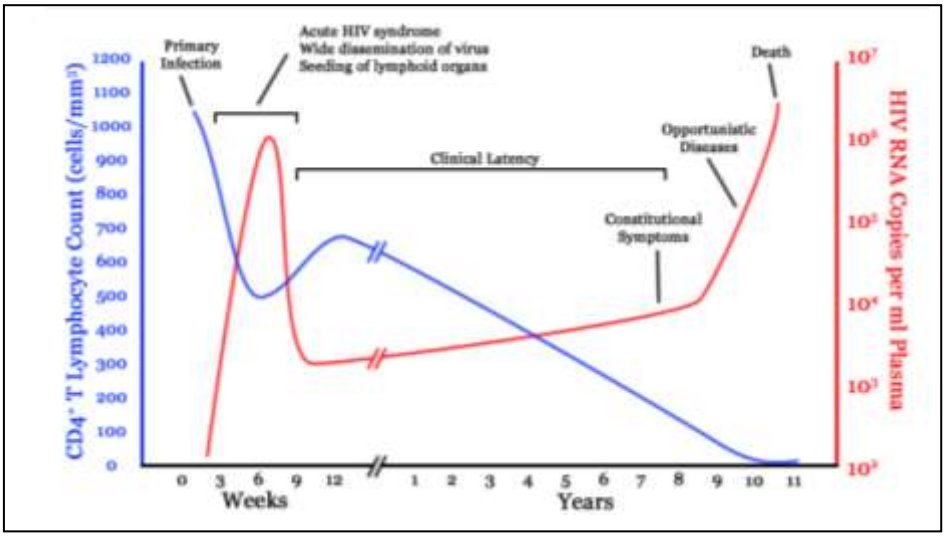

Figure 1. Typical course of untreated HIV infection - originally published in [15]

1.1.2 Human Immunodeficiency Virus (HIV)

HIV is an enveloped retrovirus, member of the genus Lentivirus and part of the family Retroviridae. Two closely related types of HIV, designated HIV-1 and HIV-2, have been identified. HIV-1 is the most common cause of AIDS, but HIV-2, which differs in genomic structure and antigenicity, causes a similar clinical syndrome. Each virion contains two copies of an RNA genome enclosed by a cone-shaped capsid of viral proteins (p24). The viral core itself is surrounded by a spherical matrix (MA) comprised of p17 proteins that are enclosed by a lipid bilayer, so called envelope (env) [18]. This envelope contains viral glycoproteins (gp120 and gp41) that bind specifically to CD4+ T cell receptors, enabling the virus to enter its host (Figure 2).

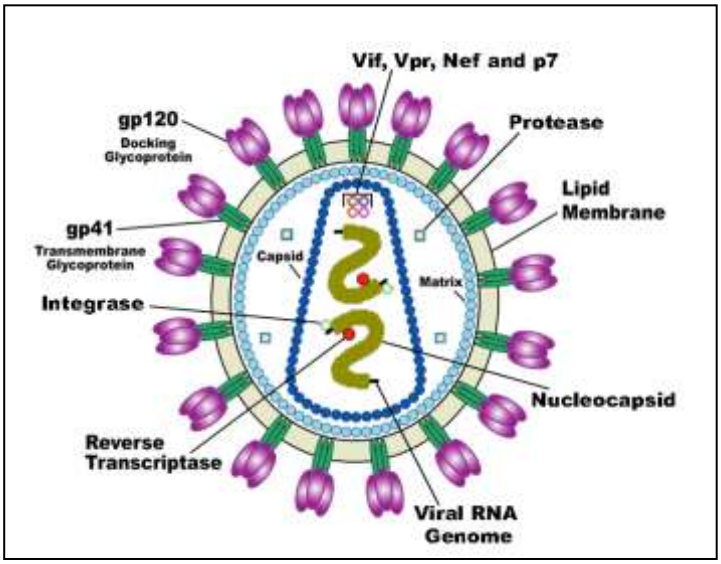

Figure 2. Structure of Human Immunodeficiency Virus type 1

1.1.3 HIV genome organization

HIV presents in its genome, many genes coding for structural proteins that are equally observed in all retroviruses, while several nonstructural ("accessory") genes are unique to HIV. Each single-stranded RNA molecule has a length of about 9.2kb and is tightly bound to nucleocapsid protein p6 and p7 [18]. Long terminal repeats (LTRs) flank each end of the genome and regulate viral gene expression, viral integration into the host genome and viral replication. HIV genes encode at least nine proteins, that can be divided in three classes: major structural (*Gag*, *Pol* and *Env*), regulatory (Tat and Rev) and accessory proteins (*Vpu*, *Vif*, *Vpr* and *Nef*). Some primate lentiviruses carry an additional accessory gene, vpx (e.g. HIV-2) or vpu (e.g. HIV-1) in the region between pol and env. The gag sequences encode core structural proteins. G*ag* precursor is cleaved by the viral protease into the mature Gag proteins, *matrix (MA, p17), capsid*

(CA, p24), nucleocapsid (NC,p7), and p6, that create a capsid that surrounds the viral genome. *T*he *env* sequences encode for glyprotein precursors gp160, cleaved by a cellular protease into two subunits of the viral spike, the glycoproteins gp120 and gp41, which are required for infection of cells. The *pol* sequences encode reverse transcriptase (RT), integrase (IN) and viral protease enzymes (PR) involved in viral replication process. In addition, HIV accessory and regulatory genes, Vif, Vpr, Vpu, Tat, Rev, and Nef, products regulate viral reproduction in various ways, but generally favoring the virus replication, packaging of the virus and the release of virions (Figure 3) [18].

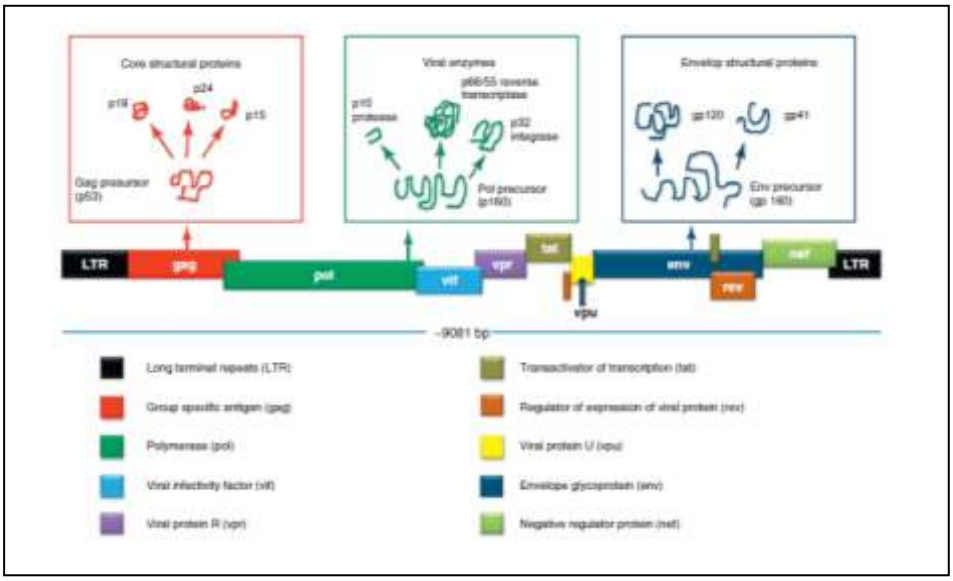

Figure 3. HIV genome structure

1.1.4 Life cycle of HIV

HIV replication can be divided in one early phase, followed by a later one [19] (Figure 4). The early phase includes the steps until viral genome integration into the host genome, while the late phase is characterized by

the production of new virus particles, in terms of HIV expression, assembly and release [18].

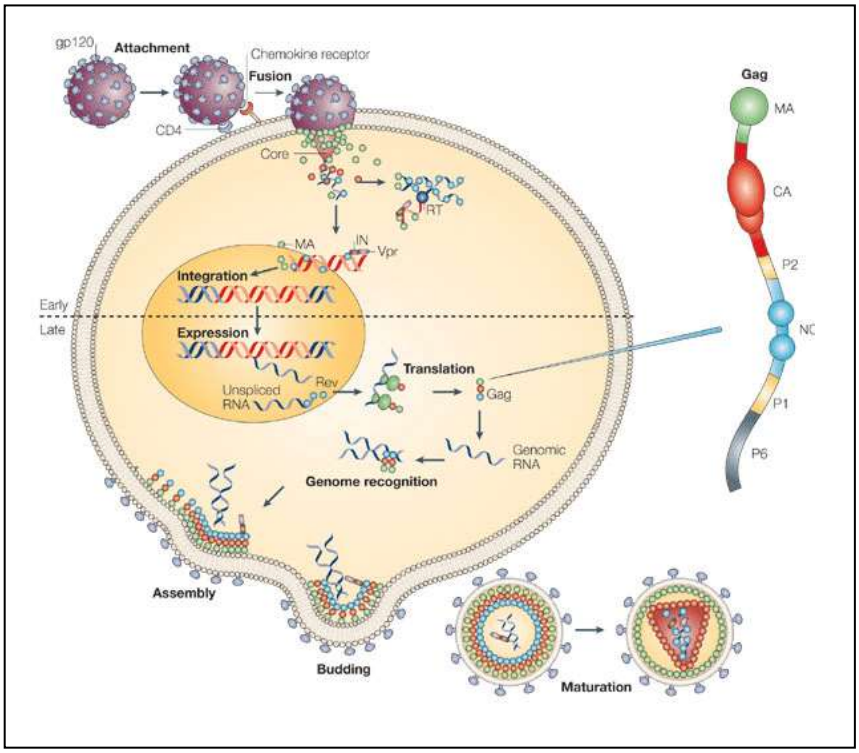

Figure 4. Replication cycle of HIV [19]

Env complex is composed by a transmembrane gp41 subunit and an external, noncovalently associated gp120 subunit. Env mediates HIV entry into target cells, promoting the binding between gp120 and the CD4 molecule on the target cells, including CD4+ T cells, macrophages and some dendritic cells. CD4 binding induces a conformational change in viral gp120 that allow the virus to interact with additional cell surface molecules, termed co-receptors, also required for entry [18].

Different isolates of HIV have distinct tropisms for different cell populations depending on the specificity of gp120 variants for different chemokine

receptors. Over 14 different seven transmembrane (7TM) receptors have been identified as potential co-receptors for HIV and SIV by their capacity to support infection of CD4+ cell lines *in vitro* [20]. The most used co-receptors are CCR5 and CXCR4, found predominantly on T cells. The HIV strains that infect primary cultures of human macrophages but not continuous T-cell lines are defined as macrophages-tropic, or M-tropic, virus and bind to CCR5, whereas the strains that infect T cell lines but non macrophages are known as T-tropic virus and bind to CXCR4. Some virus strains also infect both T-cell lines and macrophages (dual-tropic virus).

In many HIV-infected individuals, the early stages of the disease are characterized by M-tropic viruses that use CCR5 co-receptor, typically expressed on effector T cells located at mucosal sites. In contrast, during the late phases of the disease, it's typical the emergence of more virulent T–tropic viruses that preferentially bind CXCR4, mainly expressed on naïve and central memory T cells in the peripheral blood or at lymphoid sites [21, 22].

Both CD4 and co-receptor bindings induce a second conformational change in gp41, that exposes a hydrophobic region (*fusion peptide*) crucial to initiate fusion between the lipid bilayers of HIV and the cell membrane. Once HIV virions enter the target cells, the viral core translocates into the cell cytosol, where the uncoated viral genome and enzymes start the reverse transcription process. At this point, viral RNA undergoes reverse transcription, mediated by the error-prone viral reverse transcriptase enzyme, with the final production of double-stranded complementary viral DNA. Then DNA is transported into the cell nucleus, where the integration of the viral DNA into the host genomic DNA is catalyzed by the viral integrase. The HIV integrated DNA, termed *provirus*, may remain transcriptionally inactive for months or years, with little or no production of new viral proteins or virions, allowing the infection to be latent.

LTRs regulate the provirus gene transcription, containing polyadenylation signal sequences, the TATA box promoter sequence and binding sites for two host cell transcription factors, NF-κB and SP1. Therefore, any cytokines or other physiologic/antigenic stimuli capable to active T-cells and macrophages, generally enhance the viral gene transcription of HIV provirus. Upon export from the nucleus, spliced mRNA is translated to generate early gene products of the viral proteins Rev, Tat and Nef. Later in infection, these viral proteins facilitate the nuclear export of singly spliced or unspliced viral RNAs, which produce late genes, as env, gag and pol, encoding for the structural component of the virus [18].

Full-length RNA transcripts of the proviral genome are packaged within a nucleoprotein complex that includes the gag core proteins and the pol-encoded enzymes required for the next cycle of integration. New progeny virions, enclosed within a membrane (envelope), that bud from the host cell and express gp120 and gp41 on membrane, proceed to infect new cells [23, 24, 25]. This process allows HIV to infect and kill multiple immune cells, specifically causing a severe depletion of CD4+ T cells, that result to be the major host immune system target [26, 27].

In the last years, active research has been aimed at developing antiviral therapies and vaccines against HIV. However one of the most important obstacle is the complex nature of HIV that presents a real challenge for researchers. HIV reverse transcriptase is characterized by a high rate of mutation of the viral genome, due to its error-prone nature. It leads to a heterogeneous population of virions within an infected individual, creating highly diverse 'quasispecies'. Indeed, it has been assessed that in a single HIV-infected cell, the emergence of new mutations at the single base pair level is extremely high, with a rate of at least one variation in viral genome per day. [28]. This is the main mechanism that favors HIV variability within a single individual but also on a population level, as indicated by the

worldwide distribution of multiple clades and subspecies of HIV [29-31]. In particular, one of the viral region that mostly result to be susceptible to new mutations is the Env protein, that displays a crucial role in the infection of host cells, but at the same time, represents one of the major viral proteins targeted by the host immune system [32]. In this way, with this low fidelity process, HIV mutates, avoiding the pressure of the immune system and rapidly develops resistance to antiviral drugs [33]. Thus, the highly variable nature of HIV combined with the virus-mediated depletion of the cells of host immune system, make impossible to mount a functional immune response capable of defeating HIV.

1.1.5 Immune responses to HIV

The majority of HIV-infected people mount an immune response to HIV during the first few months following infection that does not differ from the other viral infections, in activating the immune system. However over the time, the HIV-specific humoral and cell-mediated immune responses result to be ineffective and generally contributing to viral escape.

Studies of plasma donors who contract HIV have showed that, after HIV transmission, the first signal of immune response is the increase in acute phase proteins, such as serum amyloid A and alpha1-antitrypsin, and multiple cytokines, coinciding with an increase in plasma viremia [34, 35]. As viremia increases, so the levels of inflammatory cytokines and chemokine do in the plasma, particularly anti-viral type-I interferons (IFNs), interleukin (IL)-15, IL-22, and the pro-inflammatory cytokines IFN-γ and tumor necrosis factor (TNF)-α. The earliest cellular sources of cytokines production include CD4+CCR5+ T cells, plasmacytoid dendritic cells (pDCs), myeloid dendritic cells (mDCs), monocytes/macrophages, natural killer /natural killer T (NK/NKT) cells and, subsequently, HIV-specific T cells [26, 34, 36]. Collectively, this early intense immune response to HIV is defined as “cytokine storm”, due to the magnitude of the response, much

higher than that observed in acute B and C hepatitis infections. Although cytokines enhance protective antiviral immune responses in acute HIV infection, the cytokine storm probably also contributes to harmful immune activation, promoting viral replication rather than clearance the virus and favoring CD4+T cells loss.

A few studies have measured that HIV specific CD8+ T cells responses occur during early infection (10-20 days post infection), before the first antibodies are detectable [37] (Figure 5). As the CD8+ T cells response approaches its peak approximately 1-2 week later, the plasma viral load declines and reach a steady set point. This suggests that anti-viral cytotoxic T lymphocytes (CTL) help control initial virus replication by directly perforin and Fas ligand based HIV-infected cells killing or by secreting soluble factors with antiviral activity [38]. Despite higher frequencies of HIV-specific CD8+ T cells in HIV-infected individuals, [39-41] suppression of viral replication is not achieved. The main reason is the emergence of viral escape mutations that allow the virus-infected cells to be hidden by host cytotoxic T-lymphocyte [42-44]. Moreover, some *in vitro* studies have revealed that HIV-specific CD8+ T cells present a reduction in their cytolytic activity [45] and poor ability of proliferation [46].

Despite the virus infects and depletes CD4+ T cells, these cells also mount an early response to HIV antigens. However, this response is dysregulated and although acts in helping for both cellular and humoral adaptive immune responses, CD4+ T cells still are particularly susceptible to HIV, remaining its preferential targets [47]. Thus, the cells that theoretically should protect individuals against virus contribute for its expansion. Potentially, in HIV-infected individuals treated shortly after infection and in a cohort of chronically infected people with ART-suppressed viremia, it has been observed that CD4+ T-cell proliferation responses to HIV antigens sometimes can be preserved or restored [48].

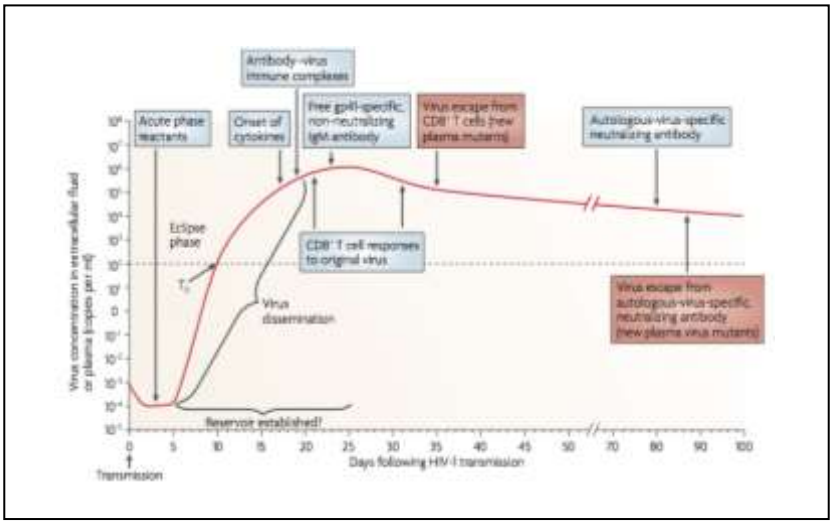

Figure 5. Earliest innate and adaptive immune responses after HIV−1 transmission [34]

Finally, the antibody response to HIV infection first occurs at approximately 13-25 days post infection in the form of immune complexes and non-neutralizing antibodies, a majority of which are specific for gp41 and gp120. The antibodies, produced early in the infection, only partially inhibit viral infectivity or cytopathic effects. Studies on viral dynamics based on mathematical modeling, have shown that produced early antibodies do not result in select for viral escape mutants. These data suggest that they resulted to be ineffective at limiting viral replication [49]. Antibodies that neutralize HIV develop slowly, around 12 weeks or longer post HIV-1 transmission. However, at this point, these more potent antibodies hardly contribute to significantly control HIV infection, due to the emergence of neutralization-resistant viral variants that evade the immune responses [50]. The rapid immune escape of HIV from anti-viral CTL and antibodies induces a compensatory increase in immune cells specific for new viral variants. The persistent viral burden and the immune responses that the

host tries to mount against the virus contribute slowly to immune dysfunction and exhaustion.

1.1.6 'Elite controllers' – HIV infected non-progressors

Although the majority of the HIV-infected patients are incapable of controlling virus replication and progress to AIDS, a small proportion (5 to 15%) of people (long term non progressors - LTNPs) with HIV are able to control HIV replication with moderate viral loads (under 10,000 copies RNA/ml blood) and CD4+ counts within the normal range in the absence of ART [51, 52]. A further subset of these individuals, termed elite controllers (EC), is able to maintain relatively stable CD4+ T cells counts and suppress viral loads to undetectable levels. Several genetic studies have identified a correlation between HIV disease progression and the presence of certain human major histocompatibility complex (MHC) alleles. In non-human primates models infected with SIV, the same correlation between disease progression and specific Mamu alleles, has been observed [53]. In particular, rapid and slow progressors showed specific HLA alleles, specifically HLA-B35 and the HLA-B7, that correspond to more rapid progression to AIDS. Interestingly, in most of the elite controllers the presence of protective MHC class I alleles including HLA B57 and B27 has been reported [54, 55]. Moreover, the majority of LTNP and elite controllers do not exhibit CCR5 Delta 32 gene deletion [56].

Several studies have reported stronger CD8 responses to HIV-gag in elite controllers. An increase of cytotoxic granules, IL-2 and IFN-g production and CD27 cell surface expression lead to high functional avidity [57, 58]. However, the observation of absence of an intense cell-mediated response to HIV in all elite controllers, suggests that other mechanisms are responsible for controlling the virus replication and delaying AIDS progression. Natural killer cells may exert an important role in elite

controllers viremia control, since it has been documented a strong association of KIR3DL1 and KIR3DS1 with HLA B57 alleles in HIV individuals [59, 60]. Neutralizing antibodies have an unclear role, as they are nearly absent in some elite controllers [61] or higher HIV-1–specific antibody responses result to be observed and associated with low viremia level in other studies [62]. Moreover, follow up of most non-progressors has revealed a possible AIDS progression [63, 64]. Maintaining control of HIV replication, even for several years, is not sufficient to avoid AIDS and opportunistic infections, due to low levels of HIV replication, viral persistence, ongoing inflammation and viral evolution.

1.2 Origin of HIV-AIDS: zoonotic transmission among species

Before the emergence of AIDS in humans in 1981, the largest and first observed phenomena of lymphoma adenopathy was recorded in 23 captive rhesus macaques (*Macaca mulatta*) and one stump-tailed macaque (*Macaca arctoides*) of the Davis - California National primate research center between 1969 and 1971 [65, 66]. However, this macaque disease characterized by signs of immune suppression and opportunistic infections, was not reported as AIDS or even immunodeficiency, since the outbreak occurred before AIDS emergence in humans.

Due to the necessity of primate centers to exchange animals for the purpose of expanding their colonies, increasing the genetic diversity in terms of scientific projects, soon the infection spread in the different Indian-origin rhesus macaque groups, generating the highly pathogenic viruses SIVmac251 and SIVmac239. The occurrence of unintentional SIV transmission inducing lymphoma happened after the experimental transfer of Sooty Mangabeys (SMs) tissues into rhesus macaques (RMs). Retrospective analyses showed that these sooty mangabeys resulted SIV positive and represented the possible source for SIV infection in macaques

(SIVmac) [66, 67]. At that time, HIV-1 had already been identified as the cause of AIDS [8], but its origin still resulted to be unclear.

Soon, in 1986, after the emergence of HIV-2 infection in individuals from West Africa [68], a closely related-virus, SIVsmm, was identified in SMs [69]. Within a very short period of time, HIV-1 and HIV-2 infections causing AIDS in humans spread and huge lentiviruses reservoirs in African monkeys were identified. Thus, simian viruses were thought to be mainly responsible for AIDS origin for their ability to cros species barriers.

1.2.1 HIV-2 origin

Molecular analyses revealed that HIV-2 originated from several zoonotic transmissions of SIVsmm to humans, virus closely related to SIVs identified also in infected macaques [70]. SIVsmm virus has been isolated and characterized from captive, free-ranging and pet [71, 72] Sooty Mangabeys (*Cercocebus atys- SMs*). SMs are mainly spread in West Africa, where SIVsmm infection is common in the wild. The natural habitat of sooty mangabeys overlaps with the geographical region where HIV-2 is endemic in humans. Indeed, in these areas, monkeys are regularly hunted for food or kept as pets, thus promoting the virus transmission through direct contact with blood or bites [72]. There are at least eight identified subtypes of HIV-2, that are only present in countries where sooty mangabeys inhabitat, explaining the less virulence compared to HIV-1 [73]. Although SIVsmm virus has crossed the species barriers several times, only subtypes A and B are represented in the HIV-2 populations, with subtype A mostly spread in Senegal and Guinea-Bissau and the subtype B in Côte d'Ivoire [74]. Other subtypes were only detected in one or few individuals.

1.2.2 HIV-1 origin

SIVcpz is a virus closely related to HIV-1: they both share the same genetic organization and contain vpu gene that is not present in most other primate lentiviruses. In the light of this, SIVcpz results to be a strong candidate for AIDS pandemic as result of cross-species transmission from wild chimpanzees (*P.t. troglodytes*) to humans [75]. SIVcpz infection is common and widespread in Central and Eastern chimpanzees (*P.t. troglodytes* and *P.t. schweinfurthii*) but absent in the remaining two subspecies (*P.t. verus* and *P.t. ellioti*), explaining the relatively scarcity of SIVcpz in the wild. Genetic analysis revealed at least four independent cross-species transmissions that give rise to the HIV-1 groups M, N, P and O, with the earliest event occurring in 1908 near modern-day Kinshasa, Democratic Republic of the Congo [76]. Two of the four HIV-1 groups proceed from *P.t. troglodytes*, in particular the M group (main) that comprises the largest group of HIV-1 accounting for the majority of infections worldwide and N group that contains only a small number of strains localized to Cameroon. The remaining two groups, P and O, have been detected in gorillas, with a prevalence of SIVgor just restricted to few sites in Cameroon. Currently, it's unclear whether gorillas were HIV-1 O source or whether both humans and gorillas were infected with a not indentified SIVcpz strain [77]. HIV-1 Group P phylogenetically clusters with SIVgor, suggesting that it is the result of a cross-species transmission from gorillas [78]

1.3 Simian Immunodeficiency virus (SIVs) and non-human primate models in HIV-1 research

Scientific interest in the study of SIVs viruses arose after the observation that the emergence of HIV-1 and HIV-2, the pathogen agents causing AIDS in humans, was the result of cross-species transmissions of SIVs from chimpanzees/gorillas and sooty mangabeys (SMs), respectively. Currently, the study of HIV origins, the characterization of SIV genome, the evaluation

of SIV disease progression in non human primate (NHP) are valuable tools for better understanding HIV pathogenesis in humans by allowing extensive *in vivo* studies to be performed in alternative preclinical models.

1.3.1 SIV genome organization

SIVs are a large group of lentiviruses that naturally infect more than 40 African NHP species. Its prevalence is variable, from 2 percent to over 80 percent in wild different species. SIVs belong to the genus Lentivirus of the family Retroviridae and infect the equine, ovine, bovine and feline families, in addition to simian species and humans. Primate lentivirus are highly divergent considering that some viral proteins share less than 30 percent amino acid identity between the different strains of SIV and HIV in terms of genome. However, many structural, molecular and biological features are in common. Both HIV and SIV lentiviral particles are surrounded by cell-derived lipid membrane with anchored viral glycoproteins and containing enzymatic proteins. Underneath the lipid membrane, a cone–shaped capsid surrounds the two copies of positive-stranded RNA associated with nucleocapsid protein and enzymes required for reverse transcription and integration. SIV genome is about 10,000 nucleotides and contains 8-9 genes that encode for about 15 different proteins. In addition to gag, pol, env, tat and rev genes, present also in HIV genome, at least three further accessory genes vif, vpr and nef are present. Moreover, vpx is characteristic of SIVs viruses that infect Papionini tribe of monkey and HIV-2; while vpu distinguishes HIV-1 and its closely related SIVs [79] (Figure 6).

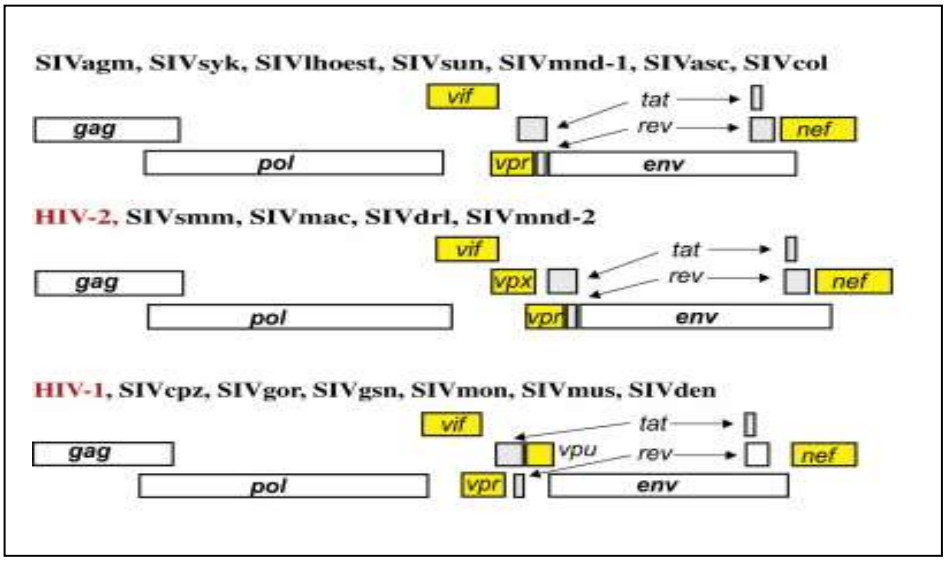

Figure 6. Genomic organization of lentiviruses [79]

1.3.2 Host Restriction factors: barriers to cross-species transmission

Viruses exploit cellular factors essential to each step of their replication in host target cells. While, mammals present the peculiar ability to use immune intrinsic factors, constitutively expressed in some cell types, to protect themselves against invading pathogens. In particular the interferon-induced antiretroviral factors that act against the virus, interfering with different replication phases, are: *TRIM5α* that binds to the incoming retrovirus capsids, recruits them to the proteasome, avoiding reverse transcription; *APOBEC3G* that inhibits reverse transcription inducing a lethal hypermutation of the viral genome and *tetherin* that 'tethers' nascent virions to cell membrane. Primate lentiviruses, from their side, exhibit the ability to develop specific tools to evade the immune system. Especially, virus accessory proteins are mainly involved in this mechanism [80]. Briefly, Vif (*viral infectivity factor*) antagonizes APOBEC3G causing its proteosomal degradation; Vpr (*viral protein R*) mediates G2 cell-cycle arrest and Vpx

(*viral protein X*) only enconded by HIV-2, SIVsmm and SIV infecting mandrillis, induces cycle arrest and facilitates macrophages infection [81, 82]. In addition, Nef (*negative factor*) and Vpu (*viral protein U*) accessory proteins display relevant roles. The acquisition of Vpu gene, only present in HIV-1 and its closest SIV counterparts, resulted in the evolution of primate lentiviruses with increased virulence and infection associated with higher immune activation levels [83]. Vpu acts enhancing the virions release by antagonizing 'tetherin' and modulating adaptive immune responses through CD4 receptor degradation [84,85]. Furthermore, the mechanism used by Vpu to affect the levels of virus-induced activation, may be affecting Nef function. It has been observed that Nef (*negative factor*), encoded by all HIV and SIV strains, down-modulates CD4, MHC-I and less efficiently CD28 and CXCR4 [86, 87], facilitating their degradation and avoiding massive T cell immune activation. In natural host of SIV, the Nef-mediated suppression of immune cell responses may be beneficial for promoting virus spread and furthermore, at the same time, reduced T cells activation may promote less viral replication-associated damage, maintaining more functional immune responses. HIV-1 Nef proteins differ from those of SIVagm, SIVsmm and HIV-2, with consequent strong response to stimulation, rendering T cell hyper-responsive. Vpu acquisition may have facilitated the evolution of viruses with an enhancement of T cell activation, explaining the acceleration of disease observed in HIV-1 infected humans [88].

The ability of the lentiviruses to rapidly mutate and evolve, adapting themselves to a new environment and host factors, qualified them for efficient cross-species transmission.

1.3.3 Viral coreceptor tropism

HIV and SIV Env glycoprotein interacts with CD4 receptor and a seven transmembrane G-protein-coupled chemokine co-receptor as entry factor. CD4 is mainly expressed on T helper cells and, at lower levels, on macrophages and dendritic cells surfaces. As in HIV infection, CCR5 is the main coreceptor used by most of the SIVs viruses to infect African NHPs. A wide variety of coreceptors repertoire, such as CCR1, CCR2b, CCR3, CXCR6, CCR8, GPR1, GPR15/Bob, STRL-33/Bonzo and Chem23 display important role for virus entry, at least in cell culture [89, 90]. However, SIVs viruses can replicate efficiently in some natural hosts, without using CCR5 or CXCR4, as occurs in SIVrcm that uses CCR2b as a major entry factor [91]. Moreover, recently, it has been identified that a subset of SMs are homozygous for a 2 bp deletion (Δ2) in CCR5, resulting in a truncated molecule not expressed on cell surface [92]. These SMs showed viral loads moderately reduced viral loads compared to the SMs with wild-type CCR5 alleles, but the presence of viremia is due to SIVsmm strains ability to use alternative coreceptors, such as CXCR6, GPR15 and GPR1 [89, 90]. It is well know that in 50% of HIV-1 infection, the progression to AIDS is characterized by a switch in viral tropism from CCR5 to CXCR4 viruses associated with a rapid decline in CD4+ T cells counts, accelerated disease progression and very poor prognosis without antiretroviral therapy [93]. In NHP models, CXCR4-tropic viruses emerge rarely [94, 95]. Interestingly, it has been reported the occurrence of SIVsmm capable to infect the host cellular targets by using CXCR4 in a cohort of SMs with loss of CD4+ T cells but with no development of any opportunistic infections or disease signs. Recent data [95] suggest that an AIDS free-status in low-CD4 SMs is possibly due to the presence of CD4/CD8 double negative T cells, particularly resistant to SIV infection with the ability to functionally compensate the lack of CD4+ helper T cells. Thus, although CXCR4 has been experimentally shown to be an effective coreceptor in SIV infection,

it's largely unclear why CXCR4-tropic viruses more frequently emerge in HIV infection than in natural hosts SIV infection. A possible explanation may be that the CD4+T cells homeostasis is preserved in SMs that maintain regular levels of CCR5+ T cells throughout the course of natural SIV infection.

1.3.4 SIV pathogenesis in natural hosts

Over 30 NHPs species of both wild and captive primates in Sub-Saharan Africa are naturally infected with SIV and are defined SIV natural host [96, 97, 98]. The first species identified as natural carriers of SIVs were Sooty Mangabeys (SMs – *Cercocebus atys*), African green monkeys (AGMs - *Cercopithecus aethiops*), mandrills (MNDs – *Mandrillus sphinx*). In SMs, AGMs and MNDs, SIV infection has been identified to be generally non pathogenic with no significant consequence of lentiviral replication in these species. Interestingly, natural hosts are able to avoid progression to AIDS, despite active viral replication of SIV at levels comparable to those found in HIV patients [99] In contrast, in the case of the pathogenic SIV infection, which primarily involved the Asian rhesus and pig-tailed macaques, infection with SIV results in a disease course very similar to HIV-1.

In African NHPs, SIV infection is horizontally transmitted and it usually occurs after sexual contacts or bite wounds [100]. SIV vertical transmission seems to be rare and the possible way of transmission (in utero, perinatally, or via breast milk) has not been identified yet. The average of SIV seroprevalence of AGMs, SMs and MNDs in the wild has been documented to be around 40-50 percent [100]. SMs are geographically distributed in coastal forests of West Africa, region of HIV-2 spread; AGMs inhabit sub-Saharan Africa and the Caribbean islands, while MNDs are endemic in Gabon and Central Africa. Among the natural hosts species that remain

AIDS-free, sooty mangabeys are the most studied as identified as the original host of HIV-2.

1.3.4.1 Sooty Mangabeys: natural history of SIV infection

SIVsmm infection of sooty mangabeys occurs at sexual maturity (approximately 4-5 years of age) and is common both in the wild and in captivity [101]. SIV-infected sooty mangabeys generally maintain normal CD4+ T cell counts despite chronic high levels of virus replication [102]. The main mechanism that allow SMs to remain healthy in presence of high viral loads have not been completely elucidated, although important insights into natural infection of SIV have been carried out in the past years. One of the used approaches to better study SIV-infected natural hosts, is to compare the features of non-pathogenic infection that are similar or distinct from pathogenic infection. As observed in humans, the acquisition of SIV infection, the primary phase and the chronic asymptomatic phases are also present in SIVsmm-infected SMs and SIVmac-infected RMs. Acute SIV infection is characterized by a high peak viral load occurring between 10-17 days post-infection that coincides with CCR5+CD4+ T cells loss in mucosal tissues and blood [103, 104] in both pathogenic and non-pathogenic models.

In natural hosts, after the peak, viremia declines and reaches set-point levels, remaining stable in the chronic phase, with viral loads comparable to HIV infection in humans. Concomitantly to CD4+ T cells depletion, an increase in activated T cells is observed. After the third week of SIV natural infection, the activation levels return towards baseline levels, in a opposite trend compared to RMs. Indeed in RMs, chronic infection is characterized by high levels of immune activation, sustained levels of virus replication, progressive loss of CD4+ T cells in the periphery, with development of opportunistic infections and finally AIDS [105]. In contrast, SMs have a general lack of cell cycle dysregulation and T cell apoptosis [106, 107];

show no evidence of lymphadenopathy or clinical signs of viral infection; maintain stable CD4+ T cell counts and slight chronic T cells activation, despite sustained levels of viral replication [108] (Figure 7).

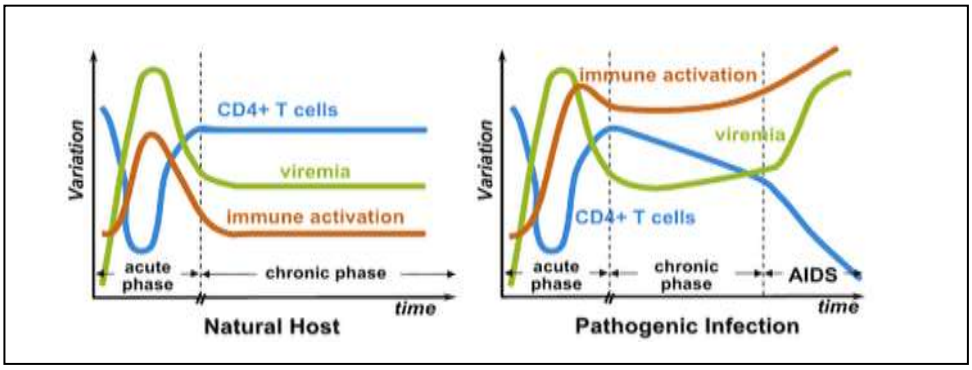

Figure 7. Pathogenic and non-pathogenic SIV infection [100]

1.3.4.2 Host cells targets and viral load

In both natural and pathogenic HIV/SIV infections, the main cell type target of infection, is represented by short-lived, activated CD4+ T cells [109,110]. CD4+ T cells display a key role in eliciting both humoral and cellular immune responses producing inflammatory cytokines and chemokines that induce immunologic activation in HIV/SIV-infection. Seventy percent of CD4+T cells, with an activated memory phenotype, are located in the gut that represents the major HIV-1/SIVmac replication site [100].

A transient leukopenia is observed during primary SIV infection of natural hosts, affecting a large number of cell subsets, and in particular CD4+T cells [111] both in blood and lymph nodes (LNs) similar to SIVmac infection. However, differently to what occurs in pathogenic models, during chronic infection, natural hosts tend to maintain close to normal levels of CD4+ T cells counts in peripheral blood and LNs, with a low number of productively infected cells [106]. In contrast, CD4+ T cells, in particular memory subsets

within the gut associated lymphoid tissue (GALT) of pathogenic models, show a dramatic and sustained depletion, with a concomitant increase of activated T cells [111]. However, the late GALT CD4+ T cells recovery is also not robust during the chronic phase in natural hosts infections, with partial restoration after one year post-infection in AGMs and SMs [111]. Moreover, a beneficial trait, that natural hosts have acquired, is the reduction in memory CCR5+ CD4+ T cell subsets, the target cells of SIV viruses, in comparison with the rhesus macaque counterpart [109]. Reduction in cells expressing CCR5 is peculiar only of natural host CD4 and not for all the memory-associated CD8+ T, that present regular CCR5 expression levels. Strikingly a recent study [112] also demonstrated that central memory CD4+ cells of SMs express less CCR5 and are less susceptible to SIV infection compared with RMs [109, 112]. The prevalence of CCR5+ CD4+ T cells in LNs explains the lower viral loads observed in SMs, that may be related to a more rapid immune clearance of virus from lymphoid tissue in natural hosts [113].

Naturally or experimentally SIV-infected natural hosts show evidence of high viremia levels, with persistent RNA plasmatic levels similar or higher than those associated with pathogenic progression [108, 100]. So the healthy status of natural hosts is not due to the presence of SIV viruses not able to efficiently infect the host. Indeed, it has been shown that SIVagm virus acquires mutations *in vivo* as rapidly as RMs, with the same potential ability to escape the immune responses as HIV-1 and SIVmac do [114].

1.3.4.3 Immune activation and HIV/SIV pathogenesis

Study of pathogenic infection has offered strong support for the role of immune activation in HIV/SIV disease progression towards AIDS [115, 116]. It has been found that high levels of pro-inflammatory plasma cytokines and chemokines, increased expression of IL-2 receptor on lymphocytes and sustained levels of activation markers, such as CD38 and

HLA-DR on CD8+ T cells, are distinctive features in HIV-infected patients, above all at more advanced stages of the disease [117]. Even patients on antiretroviral therapies (ART) with undetectable viremia, still show high T cell activation levels if compared to the healthy controls [118].

Therefore, the driving force of CD4+ T cells dysfunction and depletion, leading to AIDS in RMs and humans, is represented by the chronic immune activation as result of direct and indirect effects of the virus infection. The virus mediates the direct killing of CD4-expressing cells, leading to a homeostatic burst of proliferation, that provides effector T cells and an activated immune environment that contribute to support infection by increasing cellular targets. While, indirectly, the virus may contribute to immune activation interacting with innate immune cells, which elicit cytokines such as IFN-α and TNF-α [119]. Interestingly, SIV natural hosts maintain persistently high levels of virus in the absence of chronic immune activation [106], while the same virus results in widespread increase in immune activation in non-natural Asian macaques [96]. Strikingly, SMs mount a cellular immune response that is comparable in magnitude to the response observed in SIV-infected RMs [113, 120, 121]. The study of early events following SIV infection in SMs have revealed that the absence of chronic immune activation is a result of active down-modulation of inflammation rather than a failure in SIV-specific immune responses. In fact, SMs mount an innate immune response with a robust upregulation of type I interferon-stimulated genes (ISG) and an increase in some pro-inflammatory cytokines and chemokines, such as IP-10, IL-2, IL-6, IFN-g and MCP-1 during acute infection, similarly to what happens to RMs. By day 28, natural hosts, after a transient upregulation of these genes, show a decline in their expression, returning to baseline levels. However, in RMs the inflammation does not spontaneously resolve. In fact, in pathologic models, chronic phase is still driven by sustained and non-specific IFN-g

production [122], persistent expression of ISGs, increased expression of cells activation and proliferation markers (KI67, CD38, cell-cycling-genes) [123, 124] and markedly higher levels of CD8+ T cell proliferation [120]. The mechanisms by which SIV-infected natural hosts resolve acute phase immune activation, while HIV-infected patients and SIV-infected macaques present a persistent vicious cycle of inflammation and immune activation in chronic phases, are unclear. A possible explanation for the decline in immune system activation comes from the observation that at days 14-30 post infection, programmed death receptor-1 (PD-1) expression on SMs T cells is remarkably increased, suggesting an immune control of viral replication [125].

In addition, some hypothesize that preservation of CD4+ T cells homeostasis in peripheral blood and lymph nodes, could be a possible explanation for maintaining low levels of immune activation. However, the study of a subset of SIV-infected SMs has revealed low levels of immune activation in presence of generalized depletion of CD4+ T cells in different compartment, blood, LN, GALT, and BAL [116, 94, 126]. Therefore, preserving CD4+ T cell levels may not be a critical factor in resisting increases in immune activation.

Moreover, evidence of microbial translocation in HIV-infected patients, measured by increases in bacterial lipopolysaccharide (LPS) in the plasma, is correlated with sustained immune cell activation and elevated plasma IFN-α levels [127]. In both chronically SIVsmm-infected SMs and SIVagm-infected AGMs, microbial translocation and high LPS levels are absent [127, 128]. The balance between Th17 and Treg is preserved, contributing to maintain mucosal integrity [129, 130]. Thus, non-progressive SIV infection manifest normal lymphocytes turnover, no infiltration of CD8+ T cells into germinal centers or virus trapping on follicular dendritic cells, preservation of epithelial barrier and lack of microbial translocation [102,

108, 122, 123]. All factors that create a non-inflammatory environment that favor the absence of chronic immune activation in natural hosts.

1.3.4.4 Immune responses to SIV in natural hosts

Despite general similarities in acute phases, the major difference between pathogenic and nonpathogenic disease become evident with the occurrence of the chronic phase. Studying the transition from acute into chronic phase, could better define the roles of the different immune cell subsets in terms of contribution to resolving or potentiating a pro-inflammatory immune environment in SIV infection.

CD8+ T cells

CD8+ T-cells play a crucial role in controlling HIV/SIV replication during the infection. Several studies were able to demonstrate that CD8+T cells can efficiently inhibit viral replication *ex vivo* [131] early during infection. It has been shown a correlation between development of antiviral CD8+ T cells and post-peak viral decline [38] and that depletion of CD8+ cells in the SIV macaque models resulted in rapid increase of viral replication [132].

In natural hosts the contribution of CD8+ T cells remain to be elucidated. CD8 depletion studies resulted only in minor increases in viral load, possibly due to activation of CD4+ T cells than that absence of CD8+ T cells [133]. Moreover, in *vivo* depletion of these cells has been performed in several studies to further assess CD8+ contribution role in adaptive SIV immune responses. Transient inhibition of the CD8 lymphocyte responses resulted in a marked increase in viremia in SIV-infected RMs [131, 132]. In contrast, in AGMs, no effect on peak viral load but only a small delay in post-peak decline was observed if compared to control animals with all AGMs remained clinically healthy [134]. These findings demonstrate that delaying the initiation of CD8 + T cells responses have no impact on the

nonpathogenic outcome in SIV-infected natural hosts [100]. Moreover, investigations on the magnitude of SIV-specific CD8+ T cells responses have revealed similarities between natural hosts and pathogenic HIV/SIV hosts [120, 121, 133, 135]. Thus, it's possible that natural hosts have evolved strategy to deal with SIV infection that acts independently of CTL (Cytotoxic T-lymphocyte) responses.

Th17/ Treg

Th17 cells are a CD4+ T helper-cell subset that produces IL-17 in response to extracellular pathogens and deems critical for antimicrobial mucosal immunity. These cells are involved in the activation, recruitment and migration of neutrophils and favor the production of antimicrobial molecules and enterocytes proliferation [136]. It has been shown that this subset is depleted in the gastrointestinal tract of HIV and SIV infected individuals and rhesus, pigtailed (PTMs) macaques, respectively. In natural hosts, SMs and AGMs, SIV evolved mechanisms act to preserve Th17 cells, showing no microbial translocation or damage to the mucosal barrier, but further a functional suppression of inflammation [129, 137]. As Th17 subset is present at healthy levels in SMs, also the balance with Treg (T regulatory cells) is maintained. Treg regularly can suppress antigen-specific CD4 and CD8 responses, controlling high levels of immune activation caused by pathogens. Treg cells of SIV-infected AGMs favor the reduction in immune activation, producing IL-10, TGF-β, FoxP3 and PD1 in the early phases of infection [125, 138]. Conversely, SIVmac RMs develop this type of responses to SIV, only in later infection [125,139], not avoiding the aberrant chronic T cell hyperactivation that correlates with AIDS progression as in HIV-1 infection [122, 123].

Double Negative T cells

Double negative (DN) T cells are defined by expression of the CD3 protein, and a lack of both CD4 and CD8. Previous studies in murine models attribute to these cells regulatory functions in autoimmunity and transplantation [140]. Moreover, cross sectional analysis of DN T cells in different natural hosts species has revealed that DN T cells are found in larger proportions (10–40% of lymphocytes) in natural hosts (SMs, AGMs and patas monkeys) than in pathogenic host species (RMs). DN T cells are polyclonal and predominantly exhibit an effector memory phenotype (CD95+CD62L−). Microarray analysis of TCR (anti-CD3/CD28) in stimulated DN T cells has shown that these cells are multifunctional and upregulate genes with marked similarity to CD4+ T cells [141]. Moreover, DN T cells may have specific T helper function in a cohort of CD4-low sooty mangabeys, with no sign of AIDS progression [95]. When CD4-low sooty mangabeys were exposed to influenza vaccination, preserved immune responses were observed. These finding suggest a putative role of DN T cells in maintenance of an AIDS free status, producing Th1, Th2, and Th17 cytokines [95]. The similarities between DN and CD4+ T cells during SIV natural infection in SMs highlight a possible immunotherapeutic target to prevent HIV-induced disease progression.

B cell Responses

In the initial phase of SIV pathogenic infection, the main target of the virus is represented by T cells, but similarly, B cells are hyperactivated with subsequent massive production of virus-specific antibodies. However, in short periods, B cells encounter a rapid loss, a huge amount of CD8+ T cells migrate in the germinal centers in HIV-1 and SIVmac infections and, with the progression of the disease, the normal architecture of these centers disappear. Although the transient reduction is also observed in the

natural hosts in acute phase, B cells then are reconstituted, with no sign of lymphadenopathy, no lymphocytes sequestration into LNs or CD8+ T cells infiltration of the germinal center during chronic infection [108].

In vivo CD20+ B cells depletion studies were conducted to elucidate the role of the humoral arm in mounting an immune response against SIV. Only prolonged depletion of B cells in RMs, negatively affects the virus replication, that increases during chronic phase. If the role of the humoral responses in the pathogenic models seem to be unclear, depletion of CD20+ B cells in AGMs show minor effects on viremia with no remarkable AIDS sign [134]. Moreover, although natural hosts are able to mount neutralizing responses [142], the high plasma viremia that characterized them, explain how these responses are not more efficient than those mounted in pathogenic models.

Dendritic cells

Dendritic cells (DC) play a pivotal role in linking the innate and the adaptive immune responses, recognizing viruses or microbial components through Toll like receptors (TLRs) or other pattern recognition receptors (PRRs). When activated, DC migrate to secondary lymphoid organs where interact with different immunity cells, including NK cells, monocytes, T and B cells to mount an early immune response. In blood, among DC, the major subsets are represented by myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). pDCs, expressing TLR7 and TLR9, are one of the first responders to lentiviral infection. These cells contribute to enhance the pro-inflammatory environment, secreting IFN-α and other cytokines. During the first two weeks of pathogenic SIV infection, the levels of circulating pDCs decline in blood. Concomitant with the pDCs decrease in blood, plasma IFN-α levels and pDC numbers transiently increase in lymph nodes, where they encounter high levels of virus [143]. However, whether for natural hosts their frequencies returns to baseline levels after early phases of

infection, in pathogenic infection the decline continues, with constant pDCs recruitment in LNs favoring disease progression [144]. The control of aberrant IFN-α production by pDCs during acute infection may be critical for avoiding subsequent global immune activation during nonpathogenic infection. From the other side, mDCs are able to secrete IL-12 and IL-15, inducing T helper (Th1) cells, with subsequent activation of CTL responses that are implicated in the clearance of SIV-infected cells [145]. Moreover, IL-12 and IL-15 cytokines induce NK cells responses. In pathogenic infections AIDS progression is associated with mDCs depletion, due to their chronic recruitment to mucosal sites and their excessive cell death by apoptosis [146]. Residual mDCs from blood and intestine present an hyperactivated profile and produce pro-inflammatory cytokines in response to LPS stimuli, exacerbating the general immune activation and inflammation during SIV infection. Although AGMs circulating mDCs were initially depleted, a recovery to basal values is observed after the viral peak. In natural hosts, mDCs appear to promote tolerance to SIV in chronic phase likely due to the absence of intestinal dysfunction, microbial translocation or inflammation [146].

Natural killer cells

Natural killer (NK) cells are important component of innate immune system, implicated in the control of several viral infections. A correlation between NK cells and rate of disease progression in HIV-infected individuals has been documented [147]. Following HIV and pathogenic SIV infection, a functional impairment of NK cell compartment is observed, with a significant expansion in cytolytic subset (CD16+CD56-) and a decline in cytokine and chemokine-producing CD56+CD16- cell population [148, 149]. The data regarding NK cells variations during natural SIV infection are limited. However, CD16+CD56- cells show a higher cytolytic activity than SIV-

negative SMs or SIV-infected RMs [150]. In addition, IFN-γ and IL-2 responses to mitogenic stimulation of CD16-CD56+ NK cells from uninfected SMs are higher than that the observed in uninfected RMs [151]. Although, during acute infection an increase in cell number has been reported in both RMs and SMs [150], natural hosts display low increase in KI67-positive proliferating NK cells if compared with RMs. Moreover, SMs present a lower expression of the inhibiting receptor NKG2A in their NK cells, suggesting that the profile of activating and inhibiting receptors could be involved in the enhanced NK cell activity in natural hosts [150]. Although the role of NK cells is not fully investigated, these cells seem to be involved in the control of the early phases of SIV non-pathogenic infection.

Monocytes/Macrophages

Macrophages and their blood precursors monocytes are innate immune cells that express a number of pattern recognition receptors that enable them to detect bacterial antigens and clear microbial infections, promoting the recruitment of adaptive immune cells. Macrophages precursor is a common hematopoietic stem cell that originates in the bone marrow and differentiates into a committed myeloid cell [152]. Upon release into the blood, they are termed monocytes. Initially in the research field, they were considered only the precursors of tissue resident macrophages. While, latter studies have revealed that microbial antigens exposure or surrounding cytokines milieu influence monocytes differentiation into myeloid dendritic cells or macrophages [153]. In response to antigens, monocytes act eliminating them through phagocytosis, generation of reactive oxygen and nitrogen species and producing proinflammatory cytokines such as IL-1, IL-6, IL-8 and TNF-α [152]. In HIV-infected patients, monocytes and macrophages are found to be less productively infected by HIV with fewer than 1% of cells containing integrated proviral DNA [154]. The use of SIV-pathogenic models has partially elucidated the role of

monocytes and macrophages in the virus pathogenesis. Studies in RMs have shown that the massive blood monocyte turnover, observed after SIV infection, correlates with AIDS progression [155].

Moreover, monocytes and macrophages undergo changes in their functionality during pathogenic infections. The elevated TNF-α in the serum of HIV-infected patients, reported by different research groups, reveal that innate immune cells, such as monocytes, may produce inflammatory cytokine at high levels during pathogenic infection [156]. Peripheral blood monocytes from patients with AIDS are documented to be defective. Especially they exhibit disfunction in chemotaxis to several chemoattractants with a lowered ability to phagocytose multiple antigens/pathogens [157] and contribute to pro-inflammatory secretion that exacerbates general immune activation.

In contrast in natural hosts, little is known about monocytes-macrophages role in SIV infection. Similarly to pathogenic infection, around 1-8% of viral replication occurs in monocyte and macrophages in SIV-infected sooty mangabeys [110]. Cervasi et al., have documented that a selective depletion of SIV-infected macrophages in SMs receiving antiretroviral therapy, resulted in a delayed viral rebound after ART interruption, suppressing the level of ongoing viral replication and reducing the reservoirs of infected cells that active viral replication [158]. As CD4+ T cells, CCR5 expression on monocytes of natural hosts is also lower than in progressive hosts [109]. The potential contribution of monocytes and macrophages to the low levels of immune activation in natural hosts is still unclear and under investigation.

1.4 Antiretroviral therapy against HIV

Except for the case of Timothy Ray Brown, also known as the ‘Berlin patient’, that has been cleared by HIV, after a stem cell transplants from

one donor homozygous for the Δ32 mutation [159], there is no reported case, today, of a patient cured from AIDS. More recently, great excitement was generated for another case of the so called 'Mississippi baby', born from an HIV-positive mother in 2012. Although, an early intensive treatment was administered, last July, the virus returned and it was detected in the baby blood [160]. A safe and effective HIV vaccine remains the key requirement to better defeat the virus. In the past 30 years, several scientific efforts were realized, using whole inactivated virus, envelope subunit protein, gag, pol and nef proteins and expression vectors, from canarypox to adenovirus, aimed at eliciting B and T cells responses [161, 162]. However, none of those approaches have succeeded. Nowadays, a vaccine against HIV is still not available and, the recent found results are less than encouraging. In 2007, the highly sponsored STEP-trial proposed by Merck and aimed at testing a promising vaccine candidate in a study that saw involved more than 3.000 people, resulted to be ineffective. Merck's vaccine lacked vaccine efficacy, showing no signs of HIV-1 prevention or early viral level reduction, but moreover, it appeared to enhance infection in treated individuals with respect to the control group patients [162]. A new set of studies conducted by Hansen et al, in animal models have demonstrated how a replicating cytomegalovirus vector expressing simian immunodeficiency virus (SIV) antigens could eradicate early SIV infection in 50% of SIV-challenged rhesus macaques [163, 164]. However, the discovery that SIV eradication was associated to an unusual form of CD8+ T cells with the ability to recognize SIV peptides bind to MHC class II molecules, revealed that a more intense research need to be done, to be able to possibly translate these findings in humans. In September 2009, a Thai vaccine trial, named RV144, revealed to have some promising results.The prime-boost vaccine was aimed at eliciting both CTL and humoral responses, through the administration of ALVAC canarypox expression construct encoding HIV Gag, Pol, and Env (prime) and

AIDSVAX gp120 protein subunit (boost). This combination resulted in a 31% reduction of new HIV infections in vaccinated volunteers (compared to placebo) [165]. Although the observed results are modest and need to be conducted further analyses to understand the immunological basis for protection against HIV, efforts in the research field are encouraging. Since currently, a prophylactic vaccine is not available, HIV therapy is based on the use of antiretroviral drugs. One of the most important advances in HIV research, is the development of antiretroviral therapies (ART) that have led to a major reduction of HIV-related mortality and morbidity, with suppression of viral load, enhancement of immune functions and clinical benefits in HIV-infected individuals [166]. Only four years after HIV isolation, the first drug, called azidothymidine (ZDV or AZT), a nucleoside reverse transcriptase inhibitor (NRTI), was approved by the U.S. Food and Drug Administration (FDA) in HIV/AIDS treatment [167]. The current antiretroviral therapies can suppress viral replication below the limit of detection, contributing to delay the disease and prolog life. However virus, even under intensified ART regimen, continues to replicate and mutate, representing an obstacle to fully HIV elimination.

1.4.1 Current antiretroviral drugs

A deeper knowledge of HIV biology, pathogenesis and viral/cellular dynamics of infection have promoted the development of ART during the last years. After ART introduction, a great reduction in illness and death among HIV-infected patients has been documented, transforming the face of AIDS in developed world. Several are the already existing approved anti-HIV drugs commonly used in clinical trials (Figure 8-9). Antiretroviral therapies act targeting the different stages of the HIV-1 life cycle and promoting suppression of the viral replication [168, 169] (Figure 10).

Despite a large number of already existing drugs, multiple ones are under investigation.

1.4.1.1 Entry and fusion inhibitors

4%-16% of the European population present a homozygous Δ32 mutation in the CCR5 gene that prevents functional expression of the CCR5 chemokine co-receptor, used by HIV-1 to enter its main targets, CD4+ T cells [170]. Individuals with this genetic defect are immune to HIV infections and show no severe side effects resulting from the non functional receptor. From this observation originated the class of entry and fusion inhibitors that are molecules acting on the first step of HIV-1 life cycle. Specifically, they interfere with the binding, fusion and entry of HIV-1 to the host cell. The only approved CCR5 inhibitor is Maraviroc. Currently different entry inhibitors targeting CCR5 and the CXC chemokine co-receptor CXCR4 are under investigation [171]. Unfortunately, the virus can escape Maraviroc drug-pressure using an alternate coreceptor CXCR4. A further mechanism of preventing HIV from entering target cells, is to inhibit the step of virus fusion with host cell membranes.

Multi-class Combination Products

Brand Name	Generic Name	Manufacturer Name*	Approval Date	Time to Approval
Atripla	efavirenz, emtricitabine and tenofovir disoproxil fumarate	Bristol-Myers Squibb and Gilead Sciences	12-July-06	2.5 months
Complera	emtricitabine, rilpivirine, and tenofovir disoproxil fumarate	Gilead Sciences	10-August-11	6 months
Stribild	elvitegravir, cobicistat, emtricitabine, tenofovir disoproxil fumarate	Gilead Sciences	27-August-12	6 months

Nucleoside Reverse Transcriptase Inhibitors (NRTIs)

Brand Name	Generic Name	Manufacturer Name*	Approval Date	Time to Approval
Combivir	lamivudine and zidovudine	GlaxoSmithKline	27-Sep-97	3.9 months
Emtriva	emtricitabine, FTC	Gilead Sciences	02-Jul-03	10 months
Epivir	lamivudine, 3TC	GlaxoSmithKline	17-Nov-95	4.4 months
Epzicom	abacavir and lamivudine	GlaxoSmithKline	02-Aug-04	10 months
Hivid	zalcitabine, dideoxycytidine, ddC (no longer marketed)	Hoffmann-La Roche	19-Jun-92	7.6 months
Retrovir	zidovudine, azidothymidine, AZT, ZDV	GlaxoSmithKline	19-Mar-87	3.5 months
Trizivir	abacavir, zidovudine, and lamivudine	GlaxoSmithKline	14-Nov-00	10.9 months
Truvada	tenofovir disoproxil fumarate and emtricitabine	Gilead Sciences, Inc.	02-Aug-04	5 months
Videx EC	enteric coated didanosine, ddI EC	Bristol Myers-Squibb	31-Oct-00	9 months
Videx	didanosine, dideoxyinosine, ddI	Bristol Myers-Squibb	9-Oct-91	6 months
Viread	tenofovir disoproxil fumarate, TDF	Gilead	26-Oct-01	5.9 months
Zerit	stavudine, d4T	Bristol Myers-Squibb	24-Jun-94	5.9 months
Ziagen	abacavir sulfate, ABC	GlaxoSmithKline	17-Dec-98	5.8 months

Figure 8. Antiretroviral drugs approved by the FDA (http://www.fda.gov/)

Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs)

Brand Name	Generic Name	Manufacturer Name*	Approval Date	Time to Approval
Edurant	rilpivirine	Tibotec Therapeutics	20-May-11	10 months
Intelence	etravirine	Tibotec Therapeutics	18-Jan-08	6 months
Rescriptor	delavirdine, DLV	Pfizer	4-Apr-97	8.7 months
Sustiva	efavirenz, EFV	Bristol Myers-Squibb	17-Sep-98	3.2 months
Viramune (Immediate Release)	nevirapine, NVP	Boehringer Ingelheim	21-Jun-96	3.9 months
Viramune XR (Extended Release)	nevirapine, NVP	Boehringer Ingelheim	25-Mar-11	9.9 months

Protease Inhibitors (PIs)

Brand Name	Generic Name	Manufacturer Name*	Approval Date	Time to Approval
Agenerase	amprenavir, APV (no longer marketed)	GlaxoSmithKline	15-Apr-99	6 months
Aptivus	tipranavir, TPV	Boehringer Ingelheim	22-Jun-05	6 months
Crixivan	indinavir, IDV,	Merck	13-Mar-96	1.4 months
Fortovase	saquinavir (no longer marketed)	Hoffmann-La Roche	7-Nov-97	5.9 months
Invirase	saquinavir mesylate, SQV	Hoffmann-La Roche	6-Dec-95	3.2 months
Kaletra	lopinavir and ritonavir, LPV/RTV	Abbott Laboratories	15-Sep-00	3.5 months
Lexiva	Fosamprenavir Calcium, FOS-APV	GlaxoSmithKline	20-Oct-03	10 months
Norvir	ritonavir, RTV	Abbott Laboratories	1-Mar-96	2.3 months
Prezista	darunavir	Tibotec, Inc.	23-Jun-06	6 months
Reyataz	atazanavir sulfate, ATV	Bristol-Myers Squibb	20-Jun-03	6 months
Viracept	nelfinavir mesylate, NFV	Agouron Pharmaceuticals	14-Mar-97	2.6 months

Fusion Inhibitors

Brand Name	Generic Name	Manufacturer Name	Approval Date	Time to Approval
Fuzeon	enfuvirtide, T-20	Hoffmann-La Roche & Trimeris	13-Mar-03	6 months

Entry Inhibitors - CCR5 co-receptor antagonist

Brand Name	Generic Name	Manufacturer Name	Approval Date	Time to Approval
Selzentry	maraviroc	Pfizer	06-August-07	8 months

HIV integrase strand transfer inhibitors

Brand Name	Generic Name	Manufacturer Name	Approval Date	Time to Approval
Isentress	raltegravir	Merck & Co., Inc.	12--Oct-07	6 months
Tivicay	dolutegravir	GlaxoSmithKline	13-August-13	6 months

Figure 9. Antiretroviral drugs approved by the FDA (http://www.fda.gov/)

Nowadays, the only fusion inhibitor available is Enfuvirtide (ENF or T-20), a synthetic peptide that avoids the conformational change that facilitates the fusion between host and viral membrane, binding gp41 subunit [172].

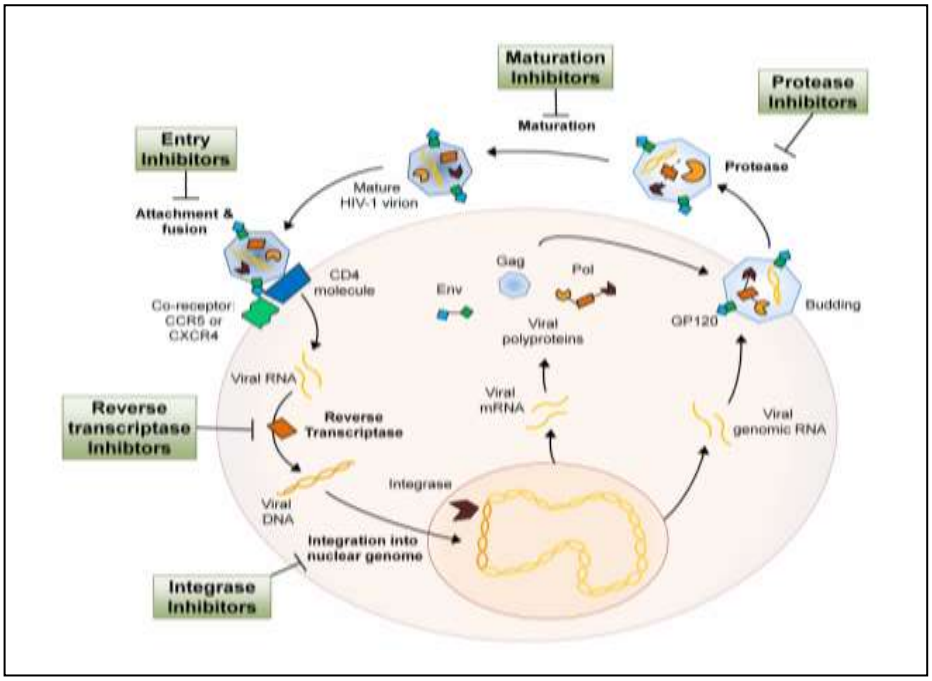

Figure 10. Antiretroviral drug classes action in HIV-1 life cycle [169].

1.4.1.2 Reverse transcriptase inhibitors

Reverse transcriptase (RT) inhibitors form the largest class of drugs against HIV. RT represented one of first target of antiretroviral therapy, interfering with the process of generating a DNA copy of the viral genome [169]. Two are the classes of reverse transcriptase inhibitors distinguished by their action mechanism. The nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) are nucleoside and nucleotide analogs that compete with their corresponding counterparts for incorporation into the newly synthesized DNA strand. These analogs lack a free 3'-hydroxyl group so

they act as chain terminators, blocking the transcription process [168, 173, 174]. Viral resistance against NRTIs is obtained by reduced susceptibility to the analogs and enhanced incorporation of the natural nucleotides or by removal of the chain terminators NRTIs at the 3' end by promoting a phosphorolytic reaction that leads to primer unblocking [174, 175]. Due to the increased adherence and better control of side effects, at least one drug of NRTIs class is commonly used in ART regimens.

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are the second group belonging to RT inhibitors class. They are polycyclic compounds that bind a hydrophobic pocket near the polymerase catalytic domain [176]. This binding induces conformational changes that alter RT flexibility, interfering with viral DNA synthesis [177]. In general, NNRTIs are safe and well tolerated and therefore usually used in first-line therapy. However, in case of mutations and lower susceptibility to one NNRTI, a cross-resistance to all the different drugs belonged to this class may be observed, with the impossibility to use other NNRTIs.

1.4.1.3 Integrase inhibitors

Integrase inhibitors (INIs), also termed strand transfer inhibitors, are a new class of drugs that aim to prevent integration of the viral DNA into the host genome acting on the enzyme integrase. INIs interfere with a specific HIV integration step. The integrase removes a dinucleotide from the long terminal repeat of each HIV-DNA strand, the enzyme cuts the cellular DNA and covalently links the viral DNA 3' ends to the target DNA [178].

Nowadays, Raltegravir, Elvitegravir and the more recently approved Dolutegravir are the available INIs. Studies on Raltegravir show its ability to reduce viral loads and increase CD4+ T cell counts if used in monotherapy and in combinations with other drugs [179]. Moreover Raltegravir has shown to be efficient in gastrointestinal tissue penetration. Raltegravir is well tolerated with a safety profile similar to that observed in the placebo

groups. It can be administered in combination with existing antiretroviral drugs, providing highly efficacious treatment to both ART-experienced and naïve subjects [180, 181]. Dolutegravir, administered once-daily, results to be as extremely active and safe as, and even more, than Raltegravir in naïve and in treatment-experienced patients [182]. Moreover, patients that have both Raltegravir and Elvitegravir resistance, show a good response after Dolutegravir administration [183].

1.4.1.4 Protease Inhibitors

Protease inhibitors (PIs) target the late phase of the viral replication, i.e. viral assembly, by inhibiting the activity of viral protease (PR), an enzyme used by HIV to cleave nascent proteins for final assembly of new virions. PIs structure is similar to the viral peptide naturally recognized by PR. These drugs act competing with the natural ligand for PR binding at the active site [184]. PIs cannot prevent the virus to integrate itself in the host genome. Thus, infected cells are replication-incompetent during a suppressive therapy but may potentially produce new viral particles in the absence of drug pressure.

Unfortunately, several reports showing resistance episodes against PIs have been documented [185]. Initially primary mutations occur near the substrate-binding cleft of the enzyme that change its structure, interfering with the binding of the PIs to the viral protease. These resistance mutations often lead to some decrease in binding affinity to the PIs, favoring the natural substrate binding [185] . Thus, they are usually accompanied by later compensatory mutations that increase the replicative capacity of the viral PR and contrast PIs action [174, 184, 186].

PIs are usually very potent both in first-line therapy as well as in subsequent treatments. Due to their reduced half-life in the body, they are usually boosted with a low dose of Ritonavir that inhibits the hepatic and

intestinal cytochrome P450 pathway involved in the metabolism of most PIs [184]. Despite suppressing successfully the virus, PIs result to have several side effects that make them replace with other drugs.

1.4.2 Viral Resistance and Highly Active Antiretroviral Therapy

The advent of ART dramatically extended the life expectancy of HIV-infected patients [166]. However, because of the extensive variability of HIV, the presence of escape mutations against individual drugs represented soon an issue. For these reasons, in 1995, the approach of combining several antiretroviral compounds marked the beginning of highly active antiretroviral therapy (HAART) era in HIV struggle. HAART combines a minimum of three drugs from at least two different drug classes to target HIV, in different phases of its replication [185]. A typical HAART regimen combines two NRTIs plus either one PI or one NNRTI [187]. The success of HAART is based on the fact that HIV has to acquire multiple resistance mutations against the different drugs included in the regimen. Immediately after HAART introduction, a rapid decline in HIV related mortality was observed [188, 189]. In HIV-infected individuals, the administration of HAART/cART resulted in a suppression of the viral replication and the preservation and limited restoration of the immune system, especially in the CD4+ T cell compartment [190]. However, these drugs are expensive and require complex daily regimens, making adherence a real challenge. Above all, this is particularly true in developing countries, where antiretroviral therapies are not available in numbers sufficient to treat those in need [191]. Strict adherence to cART is key to achieve successful HIV suppression, reduce the emergence of drug resistance, improve health, quality of life and survival, as well as to avoid HIV transmission. Furthermore, the majority of toxic side effects of these antiretroviral drugs are a serious complication, that not allow the achievement of real HAART effectiveness but rather contribute to therapeutic failure [192]. Currently a

cure for HIV is still not available and antiretroviral treatment is life long. Pharmaceutical companies aim is to improve drugs pharmacokinetics, reduce the undesired side effects of the available drugs and generate new compounds more effective at HIV elimination. Despite HAART has improved the quality of life of HIV-infected people, more efforts need to be carried out, aimed at achieving alternative and more tolerated therapies or a vaccine, in order to better treat and/or prevent HIV infection worldwide.

1.4.3 Non human primate models in anti-HIV drugs research: advantages and limits

Most of the important advances in understanding the biology of HIV infection in humans derive by the study of pathogenic models of SIV and simian-human immunodeficiency virus (SHIV) infections in Asian resus macaque, and the non-pathogenic models of SIV in African monkeys [193]. Similarities to humans in terms of physiological and immunological features, make NHP an important model for HIV investigation. The study of RM models have contributed in understanding key aspects of HIV-1 pathogenesis, especially virus transmission routes, events occurring in the post-infection phase, sites of viral replication, observation of CD4+ T cell depletion and virus/cell turnover [194, 195]. By contrast, the investigation of ART regimen effects in rhesus macaque/SIV models, is a more recent tool used in the HIV-eradication research field. [196]. This is due to the fact that SIV viruses naturally resist to NNRTIs [197] and present differences in ART pharmacokinetics if compared with humans [196, 198]. Furthermore, SIVmac is more virulent than HIV-1, showing viremia set point in SIV-infected macaques 10- to 100-fold higher than in HIV-1 infection and a more rapid progression to AIDS [199]. A recent alternative model is represented by the chimeric HIV-SIV viruses (called simian-human immunodeficiency viruses or SHIVs). Specifically, the hybrid virus is

generated by replacement of SIVmac reverse transcriptase (RT) with RT gene of HIV-1 (RT-SHIVs) *in vitro* [200, 201]. Their advantage is to be susceptible to both NRTIs and NNRTIs as occurs in HIV-infection, even if their major limit is the difficulty to completely suppress the virus with available ART regimens.

The choice of using a NHP model in eradication studies is becoming more common in the last years. The study of these models offers the opportunity to better investigate clinical parameters, hardly analyzed in humans, such as dose and route of inoculation of the virus, time of infection prior to initiation of ART, choice of the ART regimen and duration. Furthermore, extensive sampling allows investigators to characterize the cellular and anatomic compartment of both active and persistent reservoirs and, eventually, test interventions aimed at reducing viral reservoirs without exposing humans to unacceptable safety risks (i.e. stem cell and gene therapies; immune-based approaches, compounds acting at reactivating virus replication in latently infected cells) [193]. For these reasons, different combinations of antiretroviral drugs have been tested on NHP models and current therapies have demonstrated to be effective in the control of viral replication in SIV and SHIV models [200, 202, 203, 204].

It has been also highlighted how ART administration contributes to immunological recovery, increasing the CD4+ T cells levels in peripheral blood [205]. However, data regarding the reconstitution of CD4+ T cells in mucosa are discordant. Some studies suggest evidence of complete restoration in animals initiating ART during primary SIV infection [205]. By contrast, CD4+ recovery in mucosa compartment either in late or chronic infection is not observed in some NHP models [206]. In intestinal mucosa and other tissues of SIV-infected RMs, persistent viral replication can be detected even after plasma viremia suppression [207]. However, after every ART regimen interruption, in the majority of treated RMs, a rapidly increase of plasma viremia is observed, indicating the presence of residual

viral replication and latently infected cells that are not completely cleared by the current antiretroviral therapies.

1.5 HIV eradication: an ongoing challenge

In the last years, the consistent use of ART in majority of individuals, has been successful in reducing HIV-1 RNA levels to below the limit of detection, reflecting the clearance of free HIV virions and productively infected CD4+ T cells. cART has also improve the immunological HIV-induced disorders, promoting the restoration of CD4+ T cells, reducing the generalized immune activation and delaying disease progression. However, although HAART is very effective at suppressing HIV-1 replication, viral loads rebound when treatment is interrupted with AIDS progression [16, 208, 209]. So the current goal in HIV eradication research is focus on defining approaches to achieve a 'sterilizing cure' with the elimination of HIV infected cells and 'functional cure' to generate effective host immunity response that can control the virus in absence of therapies.

1.5.1 HIV/SIV latency and viral reservoirs

The major obstacle to address the formidable challenge of HIV eradication is the establishment of stable viral reservoirs of latently infected CD4+ memory T cells carrying integrated HIV-1 DNA, in the early infection phases, not affected by ART. In 2000, Siliciano et al. [210] defined a viral reservoirs as "a cell type or anatomical site in association with which a replication-competent form of the virus accumulates and persists with more stable kinetic properties that in the main pool of actively replicating virus". It has been estimated that this pool size is between 10^3 and 10^7 cells per patient, indicating that only a very small number of CD4+ T cells harbors replication-competent provirus (< 0.01%) both in the peripheral blood and lymph nodes [210, 211, 212, 213]. ART treatment led to a biphasic decay in

viremia both in HIV-infected patients and SIV-infected PMTs [214, 215]. A rapid clearance of free virions and productively infected CD4+ T cells occurs within some days after ART initiation, constituting the so called first decay phase. However, after the rapid initial decay, the subsequent proportion of cell-free virions that remain circulating in the plasma decline at slower rate. Finzi et al [216], confirmed no correlation between decrease of frequencies of latently infected CD4+ T cells and increase of time on HAART, suggesting that the observed slow decay rate is consistent with the long-term survival of resting memory CD4+ T cells in uninfected individuals. Therefore, the main contributors to virus production in the slower second phase of plasma decay are partially activated CD4+ T cells, macrophages, as well as dendritic follicular cells that gradually release trapped virions [217] (Figure 11). Nevertheless, although ART reduces the frequency of circulating resting CD4+ T cells harboring replication-competent virus to a low steady-state level in blood and tissue compartment in PMTs and humans [214, 215], residual viral replication is still present. Moreover, in 2008, Palmer and colleagues [218] further revealed the complexity of HIV persistence, characterizing furtherly the decay kinetics of viremia in a 7 years cohort of HIV patients suppressed by ART. It was found the presence of the well established first two phases, but also additional third and fourth decay phases, with 39 weeks of decay and no decay respectively were defined. The cellular sources of the third and fourth phases of decay have not been fully identified but it seems that long-lived and virus-producing cells are described as mainly contributors. One well-accepted latent cellular subtype that confers high stability to viral reservoirs, during the late decay phases, is represented by the resting long-lived memory CD4 + T cells [217].

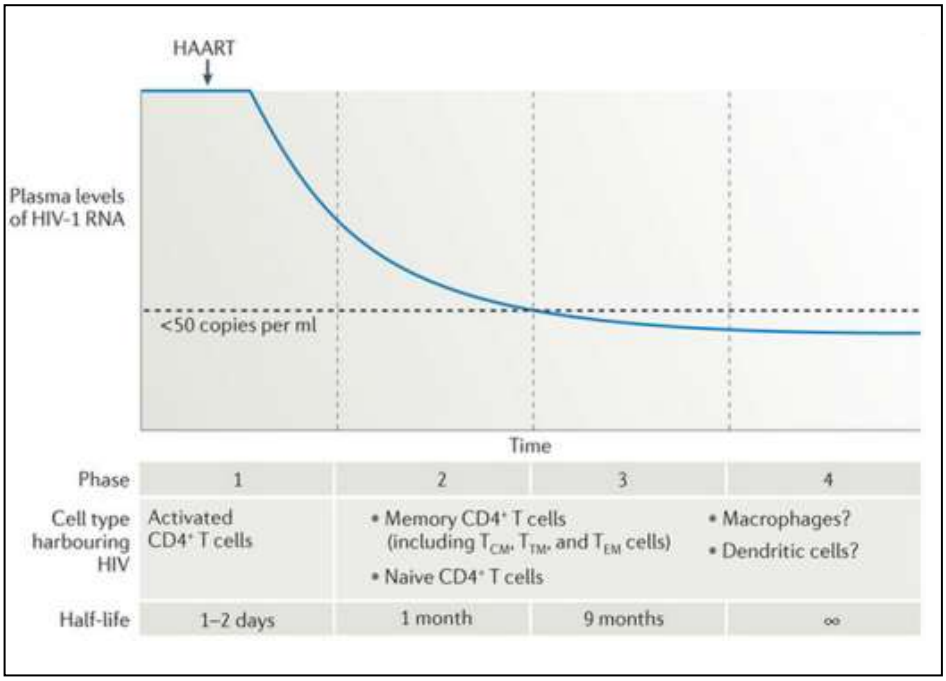

Figure 11. Impact of antiretroviral therapy on HIV reservoirs [217]

1.5.2 Cellular and anatomical sources of viral reservoirs

Nowadays, after several years of active research in this field, it's becoming clear that viral reservoirs occurred in very early phases of HIV infection [213, 219]. However, the cellular nature of latently infected cells and their exact location within the body still remain not completely understood. The best characterized cellular reservoir of HIV, at present, is a small pool of latently infected CD4+ T cells with a resting memory phenotype [210, 220, 221, 222] .

Pioneering studies demonstrated the presence of latently infected cells is thought to primarily be the result of infection of highly susceptible activated CD4+ T cells, following by their reversion to a resting state [223]. In response to antigen, resting CD4+ T cells undergo proliferation and

differentiation in effector cells. Most effector cells die but a subset survives and reverts to a resting memory state, integrating the virus and surviving for long periods [223]. Several hypotheses of virus latency has been proposed. Virus genome could possibly remain transcriptionally silent for the presence of insufficient levels of host transcription factors (PTEFB, NF-kB, NFAT and STAT5); prevalence of negative transcription factors (NF-kB1 homodimers); integration in actively or inactively transcribed genes; chromatin remodeling of HIV DNA with epigenetic silencing or transcriptional interference by host promoter activities [217, 224].

Much work has been done to further characterize the main cellular subset involved in maintenance of viral reservoirs, i.e. memory CD4+ T cells that lack of activation markers. Indeed, the memory compartment is highly heterogenous. When it has been investigated the role of different cellular subsets within the memory CD4+ T-cell pool in HIV persistence, CD4+ central memory (TCM) and transitional memory (TTM) subsets were found to be the major contributors to viral reservoirs in HIV-infected individuals on long-term ART [225]. Moreover, Chomont et al., also found that a significant fraction of latently infected CD4+ T cells co-expresses the exhaustion/inhibitory molecule PD-1. Given the assumption that the major pathway for the generation of latency is the reversion of activated cells to a resting state, it is thought that the upregulation of negative regulators of immune function and in particular PD-1, are able to aid in this reversion [225].

Moreover, more recent findings revealed that another subset of memory pool, the CD4+ T memory stem cells (TSCM), harbors high per-cell levels of HIV-1 DNA, contributing to increase the total viral CD4+ T cell reservoir over time even during suppressive ARTin HIV-infected patients [226].

There are at least three proposed mechanisms involved in the maintenance of the stable reservoir of infected resting memory CD4+ T-cells [217, 227]. First, among all, virus exploits the primary mechanisms of self-renewal of

the long-lived latently infected CD4+ T cells. In this way, these cells can intermittently release virus following homeostatic proliferation, becoming a continuous source of new infections. Furthermore, despite long-term suppression of HIV-1 replication in patients on ART, several immunological abnormalities persist and mainly an immune activated status. The presence of high levels of tissue inflammation cause the generation of activated target cells, more susceptible to HIV and responsible of cell-cell HIV spread. Finally, another possible mechanism of HIV persistence is represented by the ongoing low-levels virus replication that may occur in some anatomical sites that have unique barriers. One of ART disadvantages is the the not complete penetratation in particular sites, favoring continuous low-levels of virus replication and selection of drug-resistant strains. This mechanism may thereby be responsible for therapy failure. Among the tissues compartments relatively resistant to standard treatment, there are central nervous system, gut mucosa, tonsils, lymph nodes and male-female genital tract [217, 228, 229]. Further studies on NHP models also include additional tissues, such as spleen and liver that seem to be enriched in latently infected cells [230].

In particular, central nervous system (CNS) together with male genital tract (MGT) are considered potential sanctuary sites, for the presence of physical barriers, 'blood-testis barrier' (BTB) and blood–brain barrier (BBB) respectively [231] that render the access of anti-HIV drugs very difficult. Several short-term studies have reported a slower decay in HIV-RNA levels in cerebrospinal fluid (CSF) than in plasma, especially in patients with HIV-associated dementia (HAD). Access to the CNS by HIV-1 is mainly the result of circulating monocytes/macrophages. Same results have been found in the semen of HIV-infected individuals, where isolated T cells and macrophages harbored viral RNA [223]. Moreover, it is also thought that particularly GALT is one of the most important viral reservoirs

compartment, due to the profound degree of depletion and the high HIV proviral DNA content in central and transitional memory CD4+ T cells in treated HIV-infected patients and SIV-infected RMs [228, 232].

1.5.2.1 Non-T cell populations and SIV/HIV latency

Seminal studies recognized an additional non-T cell component involved in HIV persistence, besides memory CD4+ T cells. Indeed, it has been investigated that HIV latency may also occur in different cellular subsets, such as monocytes, macrophages, dendritic cells and hematopoietic progenitor cells [217].

Follicular dendritic cells

Follicular dendritic cells (FDCs) are localized in the germinal centers of all secondary lymphoid tissues. They are antigen presenting cells that trap and retain antigens in their native conformation, (Ags) in the form of immune complexes (ICs) formed with specific antibodies (Abs) and/or complement proteins [233]. It has been observed that HIV virions remain trapped on the surface of follicular dendritic cells (FDCs), maintaining their infectious nature without viral infection and/or replication for at least 9 months [234] *in vivo* murine models. In this way, HIV is able to persist on these cells until FDCs die. Moreover, a 2002 study has revealed that FDCs require both Ab against particle-associated determinants and FDC-FcgammaRs to maintain HIV infectivity [235].

Further studies highlighted that FDCs-trapped virus was replication-competent and genetically different from the virus isolated in other tissues and cells. In contrast, genetic similarities between FDCs – trapped virus and viral species isolated from the blood obtained at months 21 and 22 but were not present at months 4 or 18, indicating that FDCs could archive HIV [236]. Moreover, production of TNF-α by FDCs promotes a tissue microenvironment that favor HIV production and transmission [237]. For

these reasons, FDCs may be one of the possible source of low-level viremia observed during the 3rd phase of decay. The ability to retain some viral Ags for years that potentially can reignite infection after ART interruption, make speculate a possible role for these cells in HIV persistence.

Monocytes and Macrophages

The expression of low levels of CD4 on monocytes permits HIV-1 to infect this cellular subset. Indeed, monocytes circulate in the peripheral blood for less than 3 days before differentiating in macrophages. Although monocytes are more resistant to virus cytopathic effects *in vitro* than activated CD4+ T cells [238], it is well established that replication-competent virus can be recovered from circulating monocytes in HIV-1 patients, including those receiving HAART [239]. For these reasons, monocytes may represent one of the cellular subsets that contribute to viral reservoirs during of the late stages of HIV-1 disease. Furthermore, they present the ability to migrate to sanctuary sites, such as CNS and the GI, prior to maturation into macrophages, acting as vehicles of virus dissemination and providing explanation of a possible mechanism of low-viral replication in patients receiving HAART despite undetectable HIV-1 in blood [240]. Moreover, in the rhesus macaque models, circulating monocytes counts increase during the first few days following infection and the high turnover is a predictive marker of AIDS progression in SIV infection. In conclusion monocytes may play a crucial role in pathogenesis and viral persistence in HIV/SIV infection [155].

NK cells

A subset of CD56+CD16+CD3- NK cells that express both CD4 and the chemokine co-receptors CCR5 and CXCR4, resulted to be productively

infected *in vitro* by both X4 and R5 strains via a CD4-mediated mechanism. Further analysis of HIV-infected individuals on HAART demonstrated the presence of viral DNA in purified NK cells. Stimulation *in vitro* of latently infected NK cells isolated from individuals on HAART, has demonstrated virus recovery in culture [241]. It's has been observed persistent infection of NK cells and their substantial contribution to the total viral DNA in PBMCs [242].

1.5.3 Strategy aimed at eliminating viral reservoirs

Since the establishment of the viral reservoirs occurs in the first phases of the infection, different studies have evaluated the impact of early initiation of ART on viral reservoirs in both HIV-infected patients and SIV-infected RMs. Patients started on ART in the first 6 months of infection were demonstrated to have lower levels of proviral HIV-DNA in PBMCs if compared to patient who started on therapy later [243]. Moreover, nineteen patients treated during Fiebig stage I lacked detectable integrated HIV DNA in PBMCs [244]. Results were again confirmed in the french VISCONTI cohort, where 14 patients exhibited virological control following treatment cessation after 3 years of therapy with ART initiated during primary HIV infection [245]. A further case of virus replication control after treatment, was represented by the "Mississippi baby" who was started on ART within the first 30 hours of life. While early initiation of ART seems to reduce the viral reservoir size, several recent studies have shown that it is not sufficient to prevent its establishment. In particular, Louis Picker group has shown that ART initiated at day 7 or 10 after SIV infection of RMs, although causing sustained virological suppression in peripheral blood, was not sufficient to generate a functional cure [246]. Another similar study found rebound of viremia after 24 weeks of ART in RMs that initiated therapy as early as day 3 post infection, despite no SIV RNA in plasma or SIV DNA in

PBMCs were detected [247]. Unfortunately, viremia rebound was also observed in the Mississippi baby after 17 months from ART interruption.

So among the recent interventions aimed at eradicating the viral reservoirs, the “kick and kill strategy” that result in reactivation of latently infected cells in combination with ART to eliminate from a side, the HIV-infected cells by viral cytopathic effect or natural immune responses and from the other side, preventing new infections. For this purpose, early studies have shown a non specific activation of resting memory T cells with increased HIV replication when CD3 monoclonal antibodies or combination of cytokines TNF-α, IL-2, IL-6 and IL-7 were administered in HIV-infected patients [248]. Since the expression of integrated HIV-1 is inaccessible, in part as result of histone deacetylases (HDACs) action, another proposed strategy for inducing reactivation of HIV reservoirs and reducing their size, is using a class I synthetic HDAC inhibitors. This approach has been evaluated in a Jurkat cell model of latency and in resting CD4+ T cells derived from patients [249]. Among them, valproic acid resulted to be relatively weak, while vorinostat, belinostat, givinostat were more effective at targeting specific HDACs and inducing HIV expression. In addition to the aforementioned therapeutic agents, a different approach is to modify host genetics features, generating HIV-resistant CD4+ T cells. First, cells are treated with a zinc-finger nuclease that specifically eliminate or reduce the expression of HIV co-receptors CCR5 or CXCR4 *ex vivo* and then, engineered cells were transfused back into the autologous donors [250]. Preliminary findings have demonstrated a repopulation and steady increase in modified CD4+ T cells, but their beneficial role still remain unclear.

In conclusion, further investigations on HIV reservoirs localization, identification of their cellular nature using SIV models, are critical steps towards development of interventions that could induce a long-term remission or possibly eliminate the virus entirely.

Aim of the study

2. AIM OF THE STUDY

In the last years, antiretroviral therapy has led to a major reduction of HIV-related mortality and morbidity, with suppression of viral load, enhancement of immune functions and clinical benefits [166]. Indeed, HIV-1 patients on ART show a dramatic and rapid decrease in plasma viremia below the limit of detection and higher CD4+ counts [190]. However, although ART is very effective at blocking HIV spread within the body, one of the most consistent obstacles to HIV eradication is the presence of stable viral reservoirs of latently infected CD4+ memory T cells that persist, despite ART [16, 208]. The project aim is to understand the mechanisms of the complex virus-host interactions that lead to persistent infection and to achieve the ideal combination of therapies to eliminate HIV in a non pathogenic NHP model, Sooty Mangabeys (SMs), that preserves CD4+ T cells homeostasis, avoiding the AIDS progression [102]. Based on recent published data [112, 251], SMs present specific subsets of memory CD4+ T cells, i.e., the central memory and memory stem cells, that seem to be relatively resistant to SIV infection if compared the same cell type in RMs. Our hypothesis is that SMs may be particular susceptible to cART, so the use of a four-drug regimen, consisting of PMPA/Tenofovir, FTC/Emtricitabine, Raltegravir and Darunavir) in a non pathogenic model, may be functional:

1.To investigate *in vivo* and in different anatomical compartments, how active virus replication induces persistent infection,

2.To elucidate which is the main CD4+ subset involved in SIV persistence.

A deep investigation of the cART efficacy to suppress SIV infection in SMs, the evaluation of the fraction of target cells that are impacted by residual virus replication, if present, may result in a better understanding of the mechanisms by which natural SIV hosts avoid disease progression. This approach holds promise for the design of novel preventive and therapeutic approaches to HIV infection, including working towards an HIV cure.

Material and Methods

3. MATERIAL AND METHODS

3.1 Animal procedures and antiretroviral therapy

3.1.1 Ethics statement

Twelve (3 females and 9 males; average 16.8 years) chronically SIV-infected Sooty Mangabeys (SMs; Cercocebys atys), not homozygous for CCR5-null alleles and with viral load superior to 10,000 viral RNA copies/ml, were included in this study. All animals were housed at the Yerkes National Primate Research Center of Emory University, Atlanta, GA. SMs were maintained according to the Animal Welfare Act and NIH guidelines for housing and care of laboratory animals. Animal procedures were conducted in accordance with the institutional regulations and after approval by the Institutional Animal Care and Use committee (IACUC) committee at the YNPRC (IACUC protocol #2000526). Adult SMs were assigned in our study (RAAC #2013-04). Commercial dry food supplemented with fruit, was provided by the veterinary personnel and water was available ad libitum. Room temperature was maintained at 21ºC, with a relative air humidity of 50% and a 12-h light/dark cycle.

3.1.2 Antiretroviral treatment

SMs were treated with combination antiretroviral therapy (cART) regimen consisting of Tenofovir (PMPA), Emtricitabine (FTC) – two nucleoside reverse transcriptase inhibitors; Raltegravir (RLT) - an integrase inhibitor and Darunavir (DRV) - a protease inhibitor, for up to 12 months. The twelve SMs were divided in four-treatment interruption groups receiving cART for 2, 6, 9 and 12 months (Figure 12). PMPA and FTC were administered subcutaneously once a day, at the dosages of 20 mg/kg and 30 mg/kg, respectively. Stock solutions of FTC were prepared in phosphate-buffered saline (PBS, pH 7.4) at concentration of 100 mg/ml. To be at the

concentration of 80 mg/ml, PMPA was suspended in PBS with NaOH added to obtain a final pH of 7.6. FTC and PMPA stocks were filtered and stored at -20°C. RLT and DRV were administered orally (mixed with food) twice daily at the following dosages: Raltegravir, 300 mg/day; Darunavir 800 mg/day. Drug dosages were adjusted weekly according to body weight. PMPA and FTC are provided by Gilead Sciences, Inc (Foster City, CA). Darunavir was provided by Johnson and Johnson (New Brunswick, NJ) and Raltegravir was provided by Merck Sharp & Dohme Corp (Whitehouse Station, NJ).

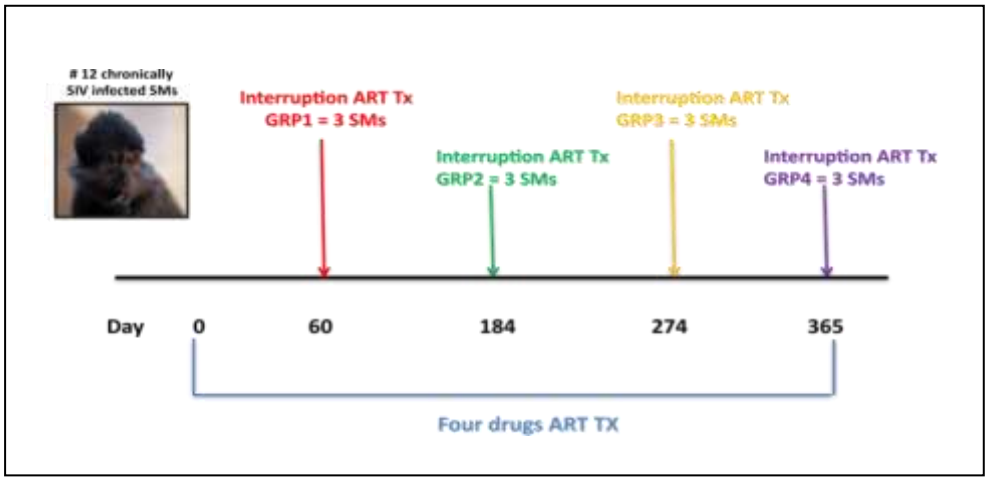

Figure 12. Study experimental design

3.2 Samples collections

3.2.1 Blood and Rectal biopsies specimen

The blood volumes that can be collected in NHPs for experimental procedures vary by species, sex and individuals, but they generally are around 8% of body weight. A maximum, safe volume for a single collection is 6-10 ml/kg. According to our proposed protocol, each SM underwent multiple blood draws and rectal mucosa biopsies (RB) at different time points before, during and at the end of treatment with cART (Figure 13).

Large amount of blood, up to 40 ml, was collected every six weeks, while the monitoring of viral load and immunophenotypic analysis were performed every 2 or 4 weeks on smaller blood volumes. For Rectal Biopsies (RB), fecal material was removed from the rectum and a rectal scope/sigmoidoscope was then placed a short distance into the rectum. Blood and RB collections were performed under sterile conditions. Sedatives, such as ketamine and telazol, were administered by intramuscular injection at the doses of 10 mg/kg and 4-5 mg/kg respectively.

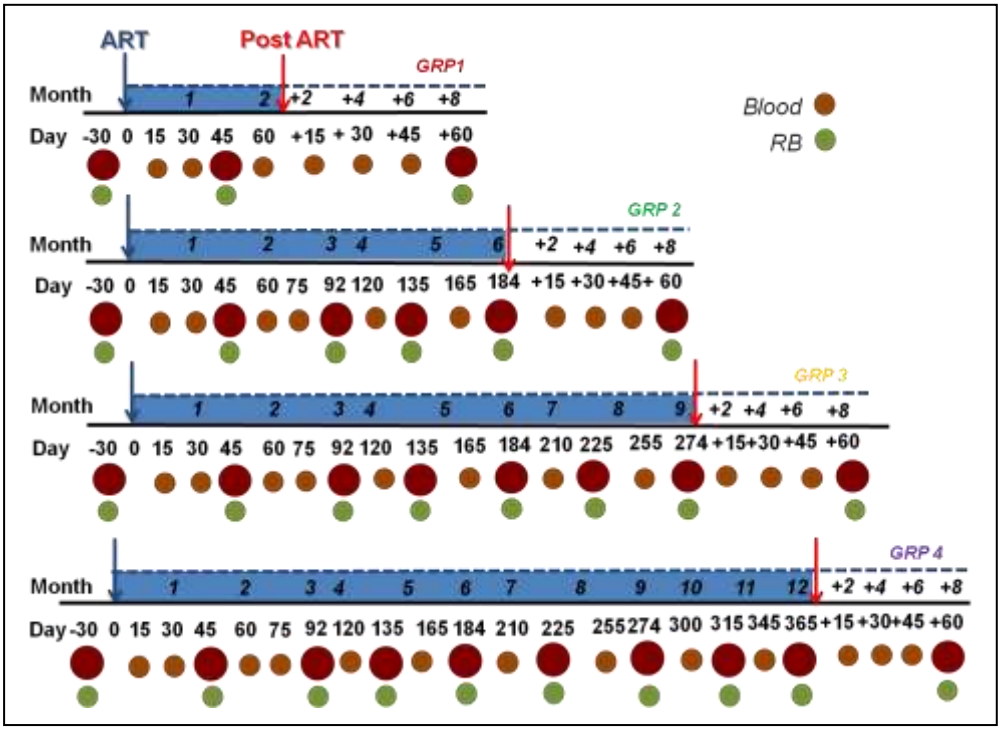

Figure 13. Blood and RB collections during the study course.

3.3 Samples processing

3.3.1 Mononuclear Cells Isolation from Blood

Peripheral blood (PB) was collected from all animals at different time points before and during the therapy by using EDTA-containing tubes. Plasma was obtained after double centrifugation at 2500 rpm for 15 minutes and then at 1800 rpm for 8 minutes. Plasma samples were stored at -80°C. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood and layered on a continuous PBS-90%-diluted Lymphocyte separation medium gradient (LSM- Lonza, Basel, Switzerland). Collected PBMCs were washed in phosphate-buffered saline (PBS, Mediatech, Inc., Manassas, VA). An additional wash with PBS and EDTA 0.5 mM (Invitrogen, Carlsbad, CA) at 200 g for 20 minutes was executed to remove platelets. After cell count performed with the automated cell counter Countess, cells were frozen in Freezing Media with Dimethyl sulfoxide 5% (DMSO, Sigma Aldrich, St.Louis, MO) and Fetal Bovine Serum (FBS, Gemini Bio, West Sacramento, CA). PBMCs were stored at -80 °C.

3.3.2 Rectal Biopsies Collections

Analysis of mucosal tissue compartment was performed on each SM. For this purpose, rectal mucosa biopsies (RB) were collected at different time points before and during cART (Figure 13). RB were obtained with biopsy forceps and placed in tissue culture fluid. Two pinches of medium size were selected and transfered in PFA 4% solution for 4 hours for further immunochemistry studies. Flash freezing of one 2 mm x 2 mm rectal pinch was performed to avoid the formation of large ice crystals that interfere with sample preservation. Vials containing rectal pinches were stored at -80 C for gene profiling analysis. Remaining pinches of RB were digested with 2-0.5 U/mg Collagenase type II (Sigma-Aldrich, Saint Louis, MO) and 10 U/µl DNase I recombinant, Rnase-free solution (Roche Diagnostic Canada,

Laval, QC, Canada), shaking at 200 rpm and 37°C for 2 hours. Following mechanical tissue digestion, the obtained cell suspension was then passed through a 70 µm cell strainer (BD Falcon™ 2350, BD Biosciences, Bedford, MA). After centrifugation at 2,000 rpm for 8 minutes, the isolated lymphocytes were re-suspended in complete RPMI1640 media. The cell count and cell viability were performed using the trypan blue dye technique. The cell concentrations were adjusted to 1×10^6 cells/ml in complete RPMI1640 media before staining them.

3.3.3 Cell count

Cell count was performed with the automated cell counter Countess™ (Life Technologies, Carlsbad, CA) that combines optic system and image analysis to automate cell counting. Countess™ counter performs viability and cell counting measurement using the trypan blue method of dead cells. Specifically, 10 ul of sample were mixed with trypan blue dye and loaded on cell counting chamber slide. The camera acquires cell images from the sample on the slide and the software automatically analyzes acquired cell images. Live and dead cell concentration/ml, total concentration/ml, viability (% live cells to total cells) are provided by the counter.

3.4 Plasma viral load analysis

3.4.1 SIVsmm RNA quantification

On plasma samples collected from peripheral blood, viral quantification was performed by the Virology Core of the Emory Center for AIDS Research at Yerkes National Primate Research (NIH Grant # P30-AI-50409). 150 µl of lasma was used to extract viral SIVsmm RNA using the QIAamp Viral miniRNA kit (Qiagen, Valencia, CA). 10 µl of extracted RNA were subjected to reverse transcriptase PCR using random hexamers to prime reverse transcription (Invitrogen, Carlsbad, CA). Primer and probe sequences were

targeted to the 5′ untranslated region of the SIVsmm genome, in particular as follows, the sequence of the forward primer for SIVsmm was 5′-GGCAGGAAAATCCCTAGCAG-3′; the reverse primer sequence was 5′-GCCCTTACTGCCTTCACTCA-3′ and the probe sequence was 5′-AGTCCCTGTTCRGGCGCCAA-3′ as previously described [112].

SIV RNA copy number was determined by comparison to an external standard curve consisting of virion-derived SIVsmm RNA. For graphical reasons, samples with undetectable SIV RNA, below the limit of the assay sensitivity (60 copies per ml), were assigned a level of half of the lower limit of detection.

3.5 Flow cytometry

3.5.1 Immunophenotyping of T cell subsets

Complete blood counts and flow cytometry analysis were carried out on collected blood samples at different time-points throughout the course of the study. Fourteen-parameter flow cytometric analysis was performed on whole-blood using panels of monoclonal antibodies that are cross-reactive with SMs. To study dynamics of CD4+ and CD8+ lymphocytes, pre-determined optimal concentrations of the following antibodies were used: anti-CD3-allophycocyanin (APC)-Cy7 (clone SP34-2), anti-CD95-phycoerythrin (PE)-Cy5 (clone DX2), anti-CD62L-PE (clone SK11), anti-CCR5–APC (clone 3A9), anti-CD45RA–FITC (clone L48), anti-CCR7–PE-Cy7 (clone 3D12) (all from BD Bioscience, San Jose, CA); anti-CD28-PE Texas Red (clone CD28.2) (from Beckman Coulter, Brea, CA); anti-CD8-BV711 (clone RPA-T8), anti-CD4-BV650 (clone OKT4), anti-PD1-BV421 (clone EH12.2H7), anti-CD27-BV605 (clone O323) (from BioLegend Inc, San Diego, CA). Activation and proliferation markers on CD4+ and CD8+ T cell populations were assessed. Specifically anti-HLADR-peridinin chlorophyll protein (PerCP)-Cy5.5 (clone G46-6) and anti-Ki-67–Alexa 700

(clone B56) (all from BD Biosciences, San Jose, CA) monoclonal antibodies were used. Flow cytometric acquisition was carried out on a LSRII flow cytometer driven by the FACS DiVa software package (BD Biosciences)

3.5.2 Whole Blood staining

In order to determine the expression of specific markers on the populations of our interest, immunophenotypic analyses were carried out using flow cytometry. Whole blood was first incubated following addition of the anti-CCR7–PE-Cy7 antibody at 37°C, for 30 minutes. Proper antibodies were added for 30 minutes at room temperature (RT), then blood was lysed (FACs lysing solution, BD Biosciences, diluted 1:10 with deio water) and washed in homemade FACs Buffer (1X PBS, 0.5% BSA, 0.01% Na Azide). Cells were permeabilized with a methanol-based method, that required the use of FACs PERM (FACs Perm solution, BD Biosciences, diluted 1:10 in deio water) for 12 minutes at room temperature. After a wash with FACs Buffer, blood samples were stained with antibodies of intracellular transcription factors of interest and fixed in 1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS. Each sample was acquired on LSRII (BD Bioscience, San Jose, CA, USA) with a minimum of 120,000 collected events in the CD3 gate and analyzed with FlowJo (Tree Star Inc., Ashland, OR, USA).

3.5.3 Staining of PBMCs recovered by rectal biopsies

Cells Suspension, obtained from RB, were stained with diluted 1:10 LIVE/DEAD fixable dead cell stain in violet (Invitrogen, Carlsbad, CA) for 7 minutes at room temperature. Cells were incubated with anti-CCR7–PE-Cy7 antibody at 37°C, for 30 minutes and then the mixture of the marker of interest was added. See 3.5.2 paragraph for details about the method.

3.6 Isolation of CD4+ T cells subsets

3.6.1 Enrichment of CD4+ T cells by magnetic bead sorting

Upon PBMCs were isolated from whole blood by LSM gradient, cells were counted to proceed with CD4+ T cells magnetic isolation. CD4+ T helper cells are isolated by depletion of non-CD4+ T cells. A CD4+ T Cell Biotin-Antibody Cocktail (Miltenyi, Auburn, CA) containing biotin-conjugated monoclonal antibodies against CD8, CD11b, CD16, CD20, CD56, and CD66abce, was added to the cells for 10 minutes at 4°C. After a wash with the buffer (PBS + 2 mM EDTA + 0.5% bovine serum albumin - BSA, pH 7.2; Miltenyi, Auburn, CA), cells were incubated with microbeads conjugated to a monoclonal anti-biotin antibody for 15 minutes at 4°C. To remove the non-specific antibody bindings, another wash with bead buffer was performed. Enriched unlabeled CD4+ T cells were obtained, allowing the cells to pass through the large selection (LS) MACS column (Miltenyi, Auburn, CA) placed in the MACS Separator.

3.6.2 Cell sorting

Living cells in suspension were physically separated by fluorescence-activated cell sorting (FACS), based on staining with dye-conjugated antibodies against cell-surface proteins of interest. Sorting of CD4+ T cell effector memory (EM), central memory (CM), transitional (TTM) and memory stem (TSCM) T cells from SMs was performed on a FACS Aria II flow cytometer (BD Biosciences). First, cells were stained with diluted 1:10 LIVE/DEAD fixable dead cell stain in violet (Invitrogen, Carlsbad, CA) and then with incubated with the proper antibodies mixture as indicated in details in paragraphs 3.5.2 and 3.5.3. Specifically, anti-CD3 Alexa 700 (clone SP34-2), anti-CCR7–PE-Cy7 (clone 3D12), anti-CD95-phycoerythrin (PE)-Cy5 (clone DX2), anti-CD62L-PE (clone SK11), anti-CD45RA-APC (clone 5H9) (all from BD Bioscience, San Jose, CA; anti-CD4-BV650 (clone

OKT4) (from BioLegend Inc, San Diego, CA); anti-CD28-PE Texas Red (clone CD28.2) (from Beckman Coulter, Brea, CA) antibodies were used in the staining procedure. Cells were initially gated on the basis of light scatter, followed by positive staining for CD3 and CD4. CD4+ effector memory (TEM), transitional memory (TTM) and central memory (TCM) cell subsets were gated on the basis of characteristic expression patterns of CCR7, CD28, CD95 and CD62L, while for CD4+ memory stem cells (TSCM) the marker of interests were CCR7, CD45RA, CD28, CD95.

3.7 Cell associated DNA assay

3.7.1 Total DNA/RNA extraction and DNA quantitative PCR

After one ml PBS wash at 700 g for 10 minutes at 4°C, sorted cells were lysed and homogenized in a highly denaturing guanidine-isothiocyanate–containing buffer, RLT plus, which, immediately, inactivates DNases and RNase and disrupt cell membranes. Then, total DNA was extracted using AllPrep DNA/RNA Mini Kit (QIAGEN) according to the manufacturer's recommendations. Sample were eluted into 40 µl of elution buffer (EB) for DNA extraction and in RNAse-free H_2O for RNA extraction. Cells were first passed through an AllPrep DNA spin column to selectively isolate DNA and and then through an RNeasy spin column to selectively obtain RNA as indicated in kit instructions.

3.7.2 Quantitative PCR for SIV gag DNA

SIVsmm RNA quantitation was performed by real-time PCR. RNA was extracted and reverse transcribed as described previously [252]. Briefly, 45 ng of DNA were loaded into a 50 µl reaction using an SIVutr primer/probe set. The same SIV-specific primers and probe were used to amplify and quantify total provirus copies of SIV, as described above in paragraph 3.4.1.

Albumin was used as an internal control to quantify cell number against an external standard curve. Albumin gene specific probe (5'-VIC-TGACAGAGTCACCAAATGCTGCACAGAA-3') and flanking primers (5'-TGCATGAGAAAACGCCAGTAA-3') and 5'-ATGGTCGCCTGTTCACCAA-3') (Applied Biosystems) were used to determinate the proportion of SIV+ cells. In particular, SIV+ cells were quantified by dividing obtained SIV DNA copy number by the albumin gene copy number. Samples with undetectable SIV DNA (i.e, 5 SIV DNA copies per 10^5 cells) were assigned a level of half of the lower limit of detection for graphical purposes. Virology Core of the Emory Center for AIDS Research at Yerkes National Primate Research (NIH Grant # P30-AI-50409) performed total DNA extraction and quantitative PCR.

3.8 Statistical methods

To assess the presence of significant changes due to cART administration in CD4+ and CD8+ frequency and absolute counts in our cohort of SMs, standard deviations and means were calculated throughout the course of the study after cART inititiation. Comparisons between the level of different markers expression on CD4+ and CD8+ populations, and in their subsets, were determined during cART versus previous time points. Statistic analysis was performed using t Tests. Specifically, un-paired t Tests, un-paired t Test with Welch's correction and Wilcoxon matched-pairs signed rank test were carried out. Variations in pre-cART cell-associated virus levels, in SMs blood, were monitored over time and statistic significance was determined using the aforesaid t T test analyses. In all cases, significance was attributed at $p<0.05$. All analyses were conducted using GraphPad Prism 5.0d.

Results and Discussion

4. RESULTS AND DISCUSSION

4.1 Animal selection criteria

Despite high viremia, SIV-infected SMs generally maintain healthy levels of CD4+ cell. However, a subset of SMs is able to be infected with multitropic SIV R5/X4/R8-using virus with a consistent and generalized loss of CD4+ T cells (5-80 cells/microl of blood) but no preogression into clinical AIDS [94]. This may be the result of a two base pair deletion in the CCR5 gene that causes protein truncation in a low group of SMs (around 8 %). Homozygous mutant animals for this delection show no expression of the CCR5 co-receptor molecule on their cell surface targets [92]. Interestingly, such animals experience SIV infection with viremia comparable to wild type counterparts. To elucidate the mechanisms involved in the reservoirs maintaince and in the light of the aforesaid results, all twenty-two experimentally SIV-infected SMs housed at the Yerkes National Primate Research Center, were screened. According to our selection criteria, SMs homozygous for CCR5-null alleles were excluded, while SMs with high count of CD4+ T cells count and viral load greater than 10,000 viral RNA copies/ml were included in the study.

4.1.1 Immunological and virologic features of SIV-infected SMs

To understand the immunologic features of chronic SIV-infected SMs, we collected blood specimens from each animal of the twenty-two chronically SIV-infected SMs of the colony. Then the following parameters were considered: CD4+ T cells count, phenotype and viral load.

CD4+ T cells percentages were obtained by flow cytometry analysis on T cell populations. Absolute CD4+ count was calculated from the CD4+ T cell percentage and the total white blood cell count during the course of the study to evaluate ART efficacy. On plasma samples, RT-PCR was carried out as described earlier. Only a small number of animals in the cohort of

SMs, housed at Yerkes National Primate Center, presented low levels of CD4+ in terms of frequency and absolute counts in addition to reduced viral loads (data not shown).

The twelve SIV-infected SMs with viral load between 10^4 and 10^5 RNA copies per ml of plasma were included in the present study. Animals with high CD4+ levels are selected compatibly to the immunological features of the SMs colony. Moreover, to ensure that all animals had endogenous CCR5, each SM included in the study, was genotyped in collaboration with Dr. Ronald G. Collman, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, (USA). No mutation in CCR5 gene was observed in the selected animals (Figure 14).

Animal code	Sex	Age (yr)	Viral load (copies/ml)	CD4 cells no	CCR5 Genotype
FCs	Female	20	80700	553.427	wt/wt
Fez	Male	14	5630	391.911	wt/wt
FFs	Male	20	47000	232.712	wt/Δ2
FHa.1	Male	10	36500	1382.277	wt/wt
FIy	Male	15	45100	1166.952	wt/Δ2
FJy	Male	15	39800	708.150	wt/Δ2
FLn	Female	23	98700	434.089	wt/wt
FSs	Male	20	107000	749.953	wt/wt
FUo	Male	22	53800	237.511	wt/wt
FUv	Male	17	19200	460.938	wt/wt
FWo	Male	21	56900	390.449	wt/Δ2
FZk	Female	25	13200	986.245	wt/Δ2

Figure 14. Features of animlas included in the study. Rationale for animal selection was viral load range, CD4 count and WT (wild type) genotype.

4.2 Antiretroviral therapy safety and tolerance

The quality of life, the health and the survival of HIV-infected individuals have remarkably improved with the introduction of anti–retroviral therapy. However, despite the successes of ART, an effective HIV cure is not available. ART remains the only source available to limit HIV infection so far, but it is a double-edged sword. Typically, standard treatments consist of three different classes of antiretroviral viral pharmaceuticals to avoid viral resistance. If HIV-infected patients strincly adhere to their treatment program, a better quality of life is experienced. Unfortunately, this is not always the case, since ART non-compliance and common clinical side effects, often mild (diarrhea, nausea, headaches) and sometimes more severe (peripheral neuropathy, lipodystrofy, liver and renal damage), can cause therapeutic failure in some HIV-infected patients.

4.2.1 Monitoring of body weights and creatinine levels

Because of clinical side effects of ART therapies, we monitored all the animals receiving drugs for cART related-adverse side effects during the course of the study. In particular a follow-up of behavioural and clinical signs (including appetite) was carried out twice daily and when the animals were sedated for blood samples collection. Body weights, hepatic and renal function indices were monitored prior to initiation and at various time points throughout the study.

As indicated in Figure 15, body weights measurement revelead the stable physical condition for all animals on cART. Only two animals (FUv and FJy) exhibited a substantial loss of body weight (15-20%) from their initial. cART administration did not impact the health status of the remaining animals as shown by only minor physiological fluctuactions (2-6%).

A major concern in all NHP models is drug toxicity. This is partially due to the elevated dosages than those typically administered in the treated HIV patients. Furthemore, in both clinical and experimental settings, PMPA-

induced renal toxicity has been clearly reported. To ensure the safety of our experimental cART regimen, blood chemistry were performed to assess creatinine levels, physiological indicator of a healthy status and preserved kidney function. The studied animals, except for FUv, presented creatinine values in the adequate or below the established ranges, showing no sign of cART-related kidney failure (Figure 16). Finally, unfortunately, one animal (FWo), has been euthanized at day 43, due to its age-related healthy conditions. In conclusion, all animals showed good healthy conditions and no remarkable therapy-dependent adverse side effects. cART resulted to be safe and well tolerated by the majority of the animals included in the study.

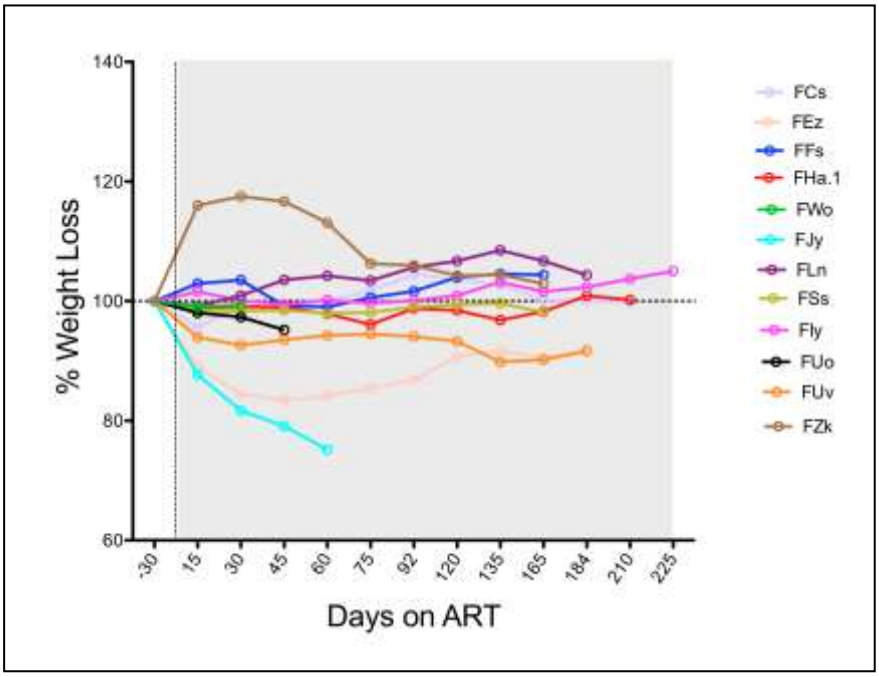

Figure 15. Effect of cART treatment on SMs weight. Loss of weight is calculated as percentage variation vs. pre-cART treatment values.

Animal codes	*Day 15*	*Day 30*	*Day 60*	*Day 120*	*Day 165*
FCs	−−	0.67	0.64	0.63	−−
Fez	1.34	−−	1.13	−−	1.05
FFs	−−	0.99	1	1.01	1.04
FHa1	−−	1.01	1.09	1	−−
Fly	0.87	0.77	0.88	−−	0.97
FJy	0.66	−−	1.58	−−	−−
FLn	1.88	−−	−−	−−	1
FSs	−−	1.07	1	0.86	1.01
FUo	−−	0.97	0.9	0.89	0.81
FUv	6.8	2.69	2.91	2.90	−−
FWo					
FZk	−−	0.78	0.7	0.67	0.77

Figure 16. Effect of cART therapy on renal function in SMs. Creatine levels were determined at various time points during the course of cART, specifically at day 15, 30, 60, 120 and 165, in treated SIV-infected SMs. Regular range: 0.8 -2.3.

4.3 New potent cART regimen for the treatment of chronically SIV-infected SMs

Antiretroviral therapy represents one of the most important findings in HIV-research of the past decade. ART is extremely effective at reduction of plasma viremia, replication suppression, enhancement of CD4+ T cells counts and improvement of life aspettance in treated patients [166, 190]. Despite ART has led to a reduction in morbidity and mortality in HIV patients, several challenges remain, including the absence of a vaccine that

can reliably prevent virus acquisition, and the inability of current ART regimens to eradicate the virus.

The treatment of HIV-infected individuals with combination of three or more drugs can suppress viral load below the clinical limit of detection (50 HIV-1 RNA copies/ml) [253]. However, residual levels of viral replication may be dectected with ultra-sensitive assays. HIV-1 persistence even with therapy, can be attributed to the presence of long-lived viral reservoirs [217]. It has been well established that viral reservoirs are mainly represented by memory CD4+ T cell subsets, which harbor silent copies of proviral DNA, that have been unable to be targeted by pharmaceuticals or the immune system [16, 208, 215].

Extensive investigation of sources of residual viremia is not flexible in human HIV infection. Therefore, it has highlighted the importance of ART-suppressed animal models to indentify stable reservoirs that contribute to viral persistence. Of particular interest to our study is the investigation of productively infected circulating populations and their anatomical localizations. Our results may be a key finding to address viral persistence in the human HIV infection.

Several groups, studying pathogenic SIV infection, have described as administration of a multi-drugs regimen, proved to fully suppress SIV and decrease memory CD4+ T-cell subsets, the main cell subsets harboring the virus *in vivo* [200, 254].

However, the effects of cART treatment on viral load and immunobiology in SIV-infected non-pathogenic NHP models are poorly understood. Previous work [110] has shown SIV-infected SMs treated with ART consisting of PMPA and FTC only. A two-phase decay of viremia was observed, with the bulk (92 to 99%) of virus replication sustained by short-lived cells (likely effector CD4+ T cells) and only 1 to 8% occurring in longer-lived cells (i.e., memory CD4+ T cells). This study suggested that in SIV-infected SMs,

similar to HIV-infected humans, short-lived activated CD4+ T cells are the main source of virus production [110].

Given these findings, we believed that our innovative experimental approach of using a potent four-drug ART regimen in a non pathogenic model represented by SMs, never conducted before, should be functional to fully achieve SIV suppression during treatment, resulting in complete eradication of residual virus production in short-lived activated CD4+ T cells.

4.3.1 Antiretroviral therapy suppresses viral replication

Twelve chronically SIV-infected SMs with viral loads ranging between 1.32 x 10^4 and 1.07 x 10^5 copies/ml of plasma were treated with a potent cART regimen, consisting of four drugs: PMPA (20 mg/kg/day), FTC (30 mg/kg/day), Darunavir (800 mg/day) and Raltegravir (300 mg/day). Plasma was collected from all experimental SMs at day 30 prior to the treatment initiation and throughout the duration of cART therapy. Sensitive RT-PCR based viral load assay was performed on plasma samples.

Interestingly, all animals receving cART experienced a rapid and significant decline of the plasma viral load. 6 out of 11 animals showed viremia suppression below the level of detection (i.e. 60 copies/ml) as early as days 15 and 30 (Figure 17). The remaining animals showed an early decline in viremia and total virus suppression at day 45 of treatment.

Only one animal (FSs) seemed to be particularly resistant to cART, experiencing undetectable plasma viremia after a prolonged treatment period (day 184) (Figure 17).

In summary, the administration of four–drugs regimen resulted to be well tolerated in all animals. cART was effective in rapid suppression of viral replication as early as 2-6 weeks in the majority of the treated SIV-infected SMs.

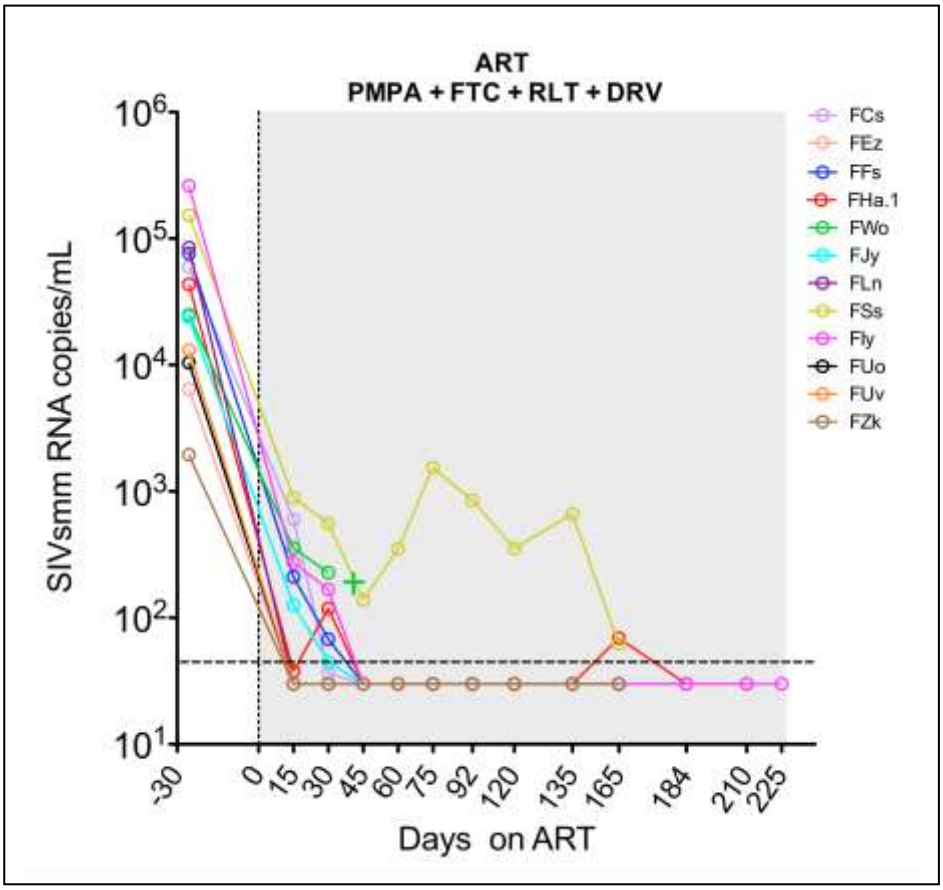

Figure 17. Effect of experimental cART regimen on plasma viremia. Plasma viral load of twelve SMs chronically infected with SIV, designated FCs, FEz, FFs, FHa.1, FWo, FJy, FLn, FSs, Fly, FUo, FUv and FZk before and during (shaded) cART. Note that animal FWo died on day 43 for ART-unrelated causes (natural death).

4.4 Immunological changes induced by antiretroviral therapy in SMs

Both untreated human HIV infection and pathogenic SIVmac infection of RMs are characterized by chronic viral replication, progressive loss of CD4+ T cells depletion and elevated levels of lymphocyte activation and

proliferation. Introduction of ART therapy induces a series of profound immunological changes in the infected hosts, consisting mainly of a rapid increase in CD4+ T cells counts and a remarkable reduction in the expression of activation and proliferation markers on CD4+ and CD8+ T cells, both in peripheral blood and in tissues [135, 190].

Specifically, the administration of ART in HIV-infected individuals can restore the pool of CD4+ T cells in a biphasic trend. The initial phase sees primarily involved the memory cells recovery, while a later phase in which naïve and other memory CD4+ subsets are restored. This is likely a result of interruption of virus-killing activity and low levels of immune activation, that permits redistribution from lymphoid tissues back to the periphery [110, 214].

4.4.1 Impact of cART on the recovery of CD4+ T cells

We first performed a cross-sectional analysis of the phenotype of T cells isolated from different anatomical compartments: peripheral blood (PB) and MALT (Mucosal associated lymphoid tissue), specifically rectal biopsy, RB. To evaluate the extent of CD4+ T cells reconstitution following cART in peripheral blood (PB), frequency and CD4+ T cell absolute counts were determined at various time points during the course of the study. Although recovery of CD4+ T cells is usually observed in SIV-infected RMs and HIV-infected humans on ART [190, 205], the analysis of total circulating CD4+ population, in all treated SMs, revealed only minor fluctuations in terms of their frequency and absolute number (Figure 18 A-B and C-D respectively). The lack of CD4+ T-cell increase in cART-treated SMs infected with SIV was not entirely unexpected, considering that non pathogenic SIV infection is not characterized by severe depletion in chronic phases but rather relatively high levels of CD4+ T cells even in absence of treatment.

Consistent with previously work [111], CD4+ T cell levels were lower in mucosal sites versus the systemic compartment prior to cART initiation (Figure 19 A). Interestingly, we observed a marked recovery of mucosal CD4+ T cells early in cART therapy as well as days 92 and and 135 in comparison to baseline levels (p= 0.0391 and 0.0078 respectively) (Figure 19 B–C). These data suggest that cART suppression in SIV-infected SMs, only plays a minor role in augmentation of the systemic CD4+ T cell compartment. While likely the lack of immune dysfunction in this species may favor homeostatic mechanisms designed to reconstitute the CD4+ T cells in the mucosa.

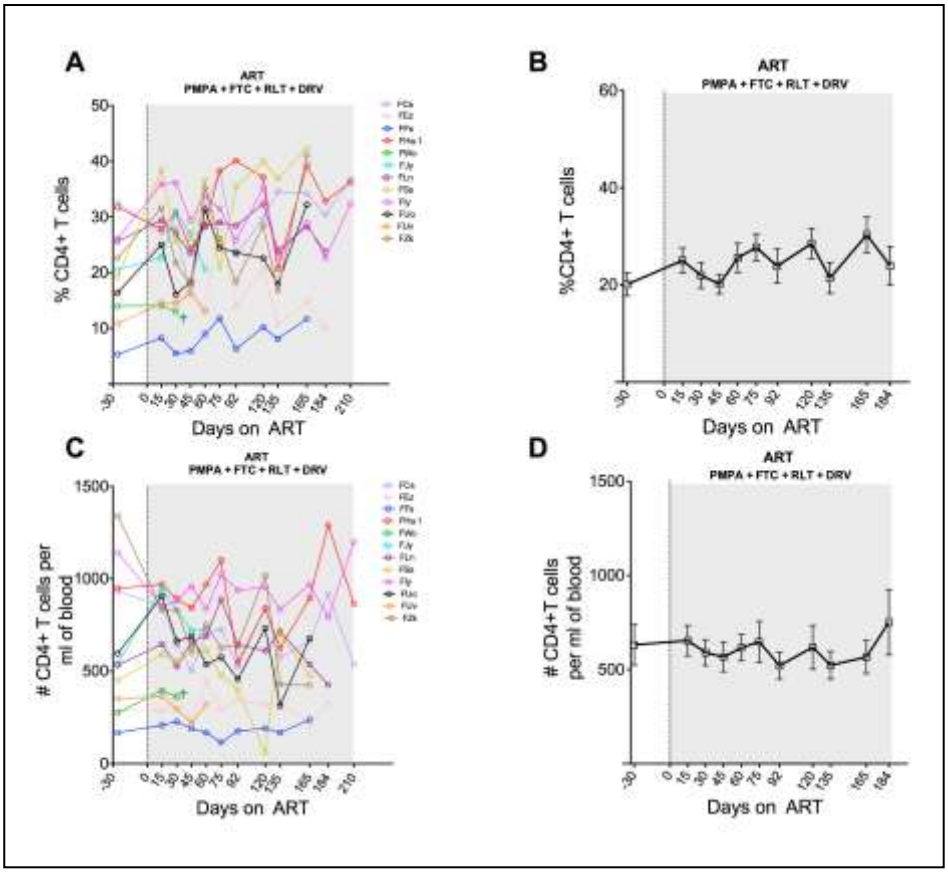

Figure 18. CD4+ T cells dynamics in peripheral blood of cART suppressed SMs. (A) Frequency of CD3+ CD4+ T cells fraction in peripheral blood (PB)

30 days before the initiation of cART and every two weeks of treatment from day 0 (shaded). (B) Average is shown in the right panel. Percentages of CD4+ T cells are determined by flow cytometry analysis on lymphocytes population. (C) CD4+ T-cell count, on the Y axis for each of the SMs before, during (shaded) the therapy, is calculated based on CD4+ T cells percentage and the total white blood cell count. (D) Average is shown in the right panel.

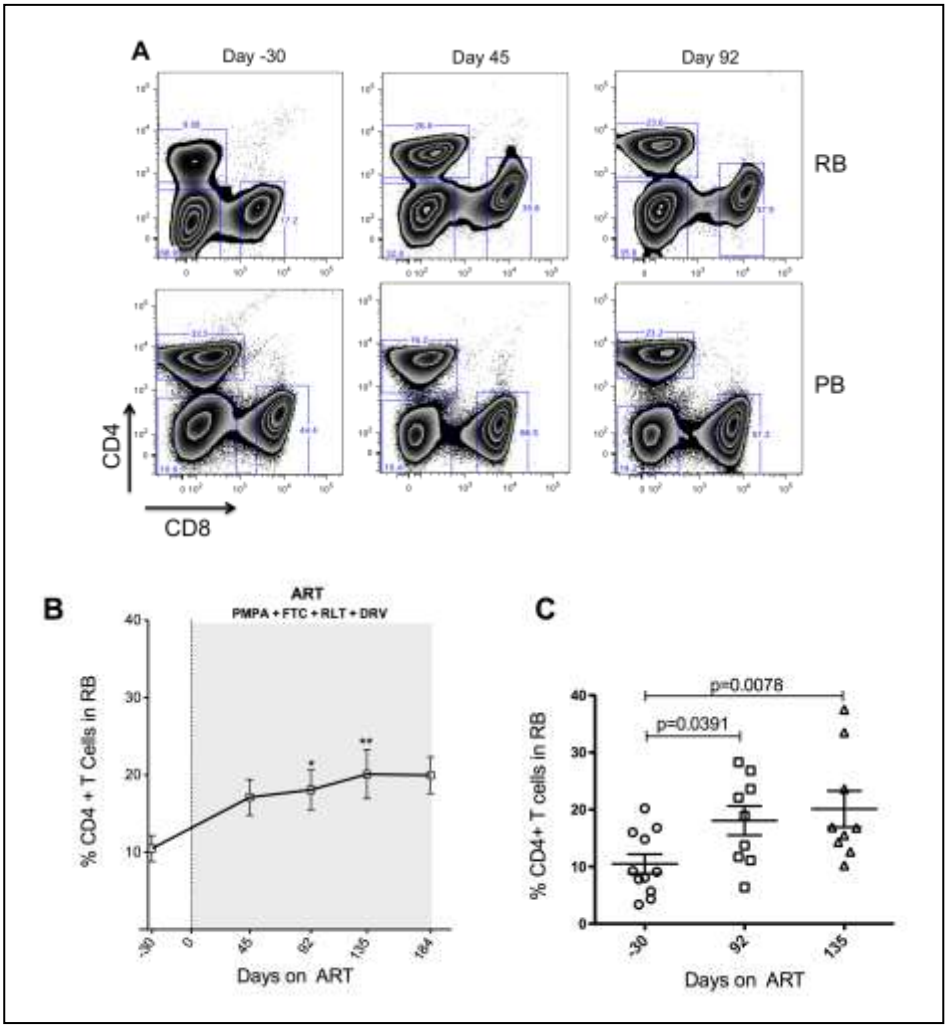

Figure 19. Impact of cART on MALT CD4+ T cells. (A) Representative flow cytometric analysis showing the fraction of CD4+ and CD8+ T cells in Peripheral Blood (PB) and rectal mucosa (RB) before cART initiation and during treatment course in one SM, gated on live CD3+ cells. (B) Average of CD4+ T cells in RB of SIV-infected SMs in absence and presence of therapy. The x-axis shows time post cART initiation (day 0). (C) Recovery of CD4+ T cells in mucosal tissue (RB). Mean values, standard deviations and statistically significant differences are shown (p<0.05, ** p<0.01 and *** p<0.001 vs baseline, day-30). Statistical significance was determined by Wilcoxon matched-pairs signed rank test.*

4.4.2 Kinetics of CD4+ T cell subsets during cART

It has been described that five species of SIV natural hosts express markedly lower levels of CCR5 on CD4+ T cells isolated from blood and lymph nodes [109]. A more in depth exploration found that long-lived CD4+ central memory (T_{CM}) are characterized by greatly reduced CCR5 expression when compared to non-natural hosts (RMs), *ex vivo* and in the context of SIV infection *in vivo* [112]. A consequence of reduced CCR5 expression is that the amount of SIV DNA found in CD4+ T_{CM} in SMs is ~1 log lower than in RMs, meaning that SMs T_{CM} are relatively resistant to SIV infection in comparison to RM T_{CM}. A more recent study [251] identified another SIV resistant CD4+ subset in natural hosts, so called T memory stem cells (T_{SCM}). Indeed, CD4+ TSCM cells exhibit substantial levels of direct virus infection in RMs measured by cell-associated DNA. While, in the majority of SMs, a complete lack of SIV-DNA detected in the same subset was observed.

Given the well-established role of memory CD4+ T cells in the maintenance of viral latency in presence of cART, we aimed to elucidate the dinamyics of memory CD4+T cell subsets during cART treatment.

Thus, a deep characterization of source(s) of viral reservoir(s) in a non-pathogenic model resistant *per se* to SIV infection may help design novel prophylactic and therapeutic interventions to cure HIV infection.

4.4.3 CD4+ T cell subsets definition

Memory T-lymphocytes can be subdivided into distinct populations. The most studied subsets are the central memory (T_{CM}) and the effector memory (T_{EM}) cells that are characterized by different homing and effector functions. Briefly, humans TCM express lymph nodes homing receptors (CD62L and CCR7); whereas TEM mainly reside in the effector sites, exhibiting β1 and β2 integrins, CCR1, CCR3, CCR5 and CD103, CLA17 markers. In non-human primate models, equivalent distinctions are made using the costimulatory molecule CD28 expression and FAS Ligand (CD95). These CD4+ T cell memory subsets are distinguished by CCR7 expression, a chemokine that modulates lymph node migration. Specifically, TEM express CD95 and low levels of CCR7; wheareas, TCM express CD95+ and CCR7 expression is high on these cells. The canonical CD45RA or the CD62L expression, commonly used in humans to define TCM and TEM subsets, have been less extensively used in non-human primate models [255].

In the light of these observations, we aimed to differently define memory CD4+ T cell subset, as a mean of more deeply understanding. To accomplish this, we used the canonical approach (CD95, CD28, CCR7) in conjunction with alternative markers. Both CCR7 and CD62L expression characterizes the fraction of TCM cells, while TEM lack the expression of both markers on cell surface.

In humans, Chomont et. Al and later Cirion et. Al., distinguished among CCR7- and CD45RA- cell fraction, another memory CD4+ T cell subset, called transitional memory (TTM), based on expression of CD27 [225, 245]. In NHP models, a definition of the pool of TTM and TCM has been

described based on CD28+CD95+ expression by Savarino [254]. To better elucidate the role of this subsest in latency maintainance, we characterized TTM as cells expressing CD28, CD95, CCR7 but not CD62L.

In the light of this new definition, twelve-parameter flow cytometric analysis was performed on whole blood before and during cART treatment. We deeply analyzed the impact of cART on different memory CD4+ T cells subsets dynamics, assessing the fraction of effector memory (TEM, CD28+, CD95+, CCR7-, CD62L-), central memory (TCM, CD28+, CD95+, CCR7+, CD62L+) and transitional memory (TTM, CD28+, CD95+, CCR7+, CD62L-) CD4+ T cells (Figure 20).

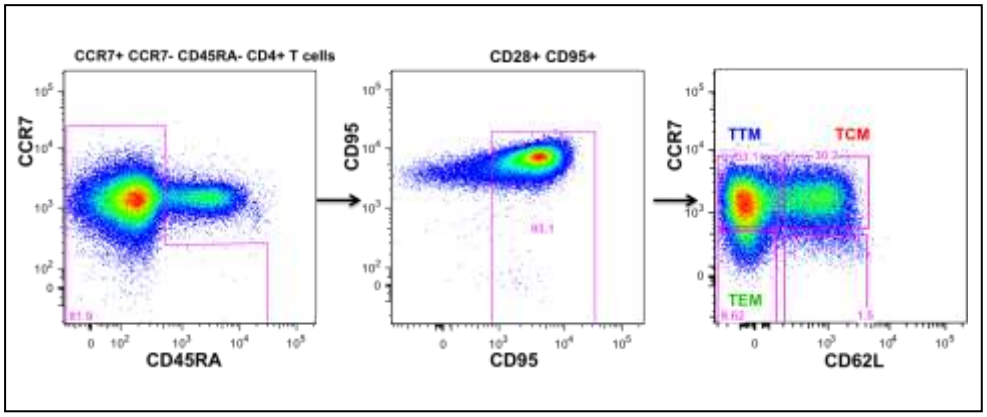

Figure 20. Gating strategy for memory CD4+ T cell subsets. Expression of the surface markers CD28, CD95, CCR7, CD62L on CD4+ T cells in a representative sooty mangabey. (A) Fractions of central memory (T_{CM}, highlighted in red), effector memory (T_{EM}, in green) and transitional memory (T_{TM}, in blue).

4.4.3.1 CD4 + Effector memory T cell subset

A rapid reduction of CD4+ effector memory T cells in terms of frequency was observed at earlier time points (day 15). TEM were significally reduced

at days 75 and 120 from the beginning of ART treatment in all animals ($p=0.0003$ and $p<0.0001$, respectively) (Figure 21 A-B). The TEM reduction in frequency was also confirmed by the absolute counts. The TEM count was significally reduced at days 75 and 120 versus baseline values ($p=0.0039$ and $p=0.0039$, respectively) (Figure 21 C-D). It has been described that TEM are short-lived cells, depleted by the virus during HIV-infection, representing the main source of viral replication [110]. Consistent with this notion, upon cART initiation, the fraction of TEM was reduced as a result of SIV-mediated death. At later time points, TEM resulted to be maintained at low levels, likely owing to cART inhibition of newly infected TEM.

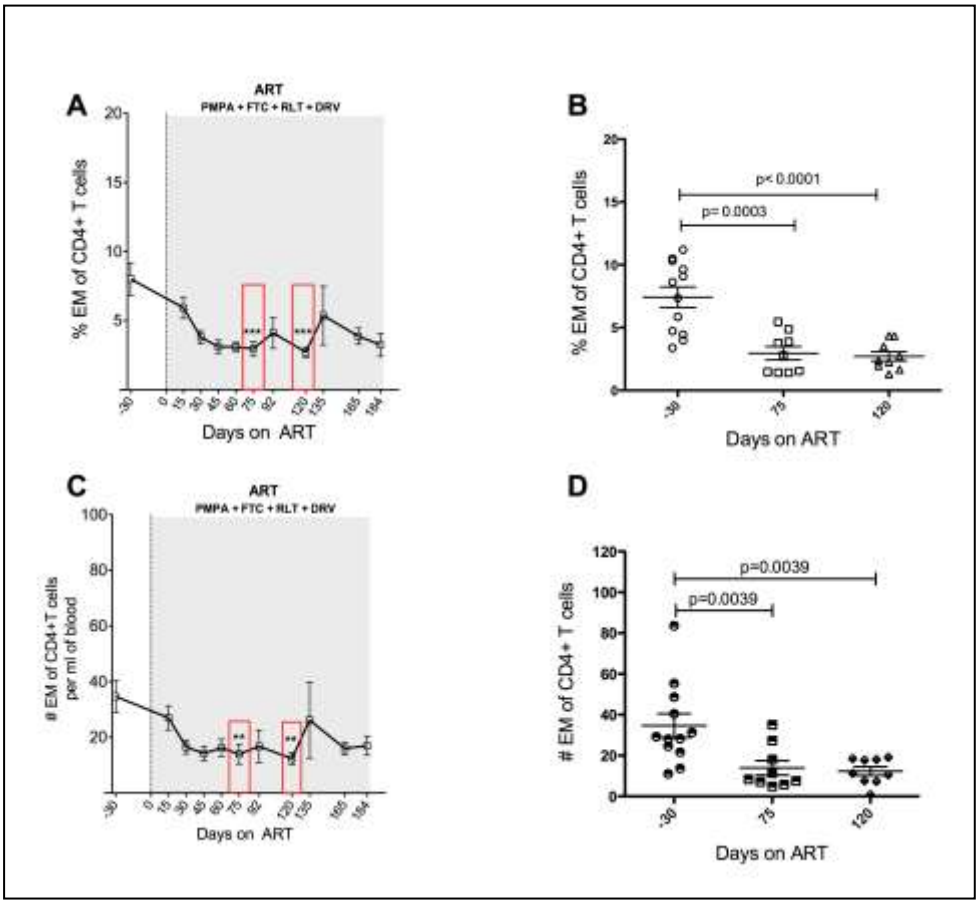

Figure 21. Longitudinal analysis of TEM CD4+ T cell subset in SIV-infected SM. (A) Average of effector memory (EM) frequency after ART initiation and significant decrease at days 75 and 120 (B). (C) Average of CD4+ TEM absolute counts and marked reduction (D) at days 75 and 120. Data were performed by flow cytometer on whole blood samples at different time points. Mean values, standard deviations and statistically significant differences are shown ($p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs baseline, day-30).*

4.4.3.2 CD4+ Central memory T cells

Recent papers showed that the tempo of disease progression in HIV/SIV infection is dictated by the depletion of long-lived TCM pool [256]. A peculiar feature of an effective ART therapy is the ability to restore immune function and enhance CD4+ T cell survival.

Interestingly, under cART regimen, we observed an expansion of central memory CD4+ T pool, at earlier time point of treatment (Figure 22 A). At days 92 and 135, frequency of TCM was significant increased ($p=0.0078$ and $p=0.0078$ respectively) (Figure 22 B). The absolute count of the CM subset was modestly elevated in cART-treated animals (Figure 22 C-D).

Our observations suggested that TCM expansion, following ART initiation, may be the result of both the interruption of virus-induced killing and the rapid redistribution of cells that were previously localized in lymphoid tissues or other effector compartments. TCM are relativey protected by reduced CCR5 expression in natural host and are restorated during cART. A viable mechanism could be that nascent memory pool generated by TCM during cART, could be harboring reduced virus or be clear of infection.

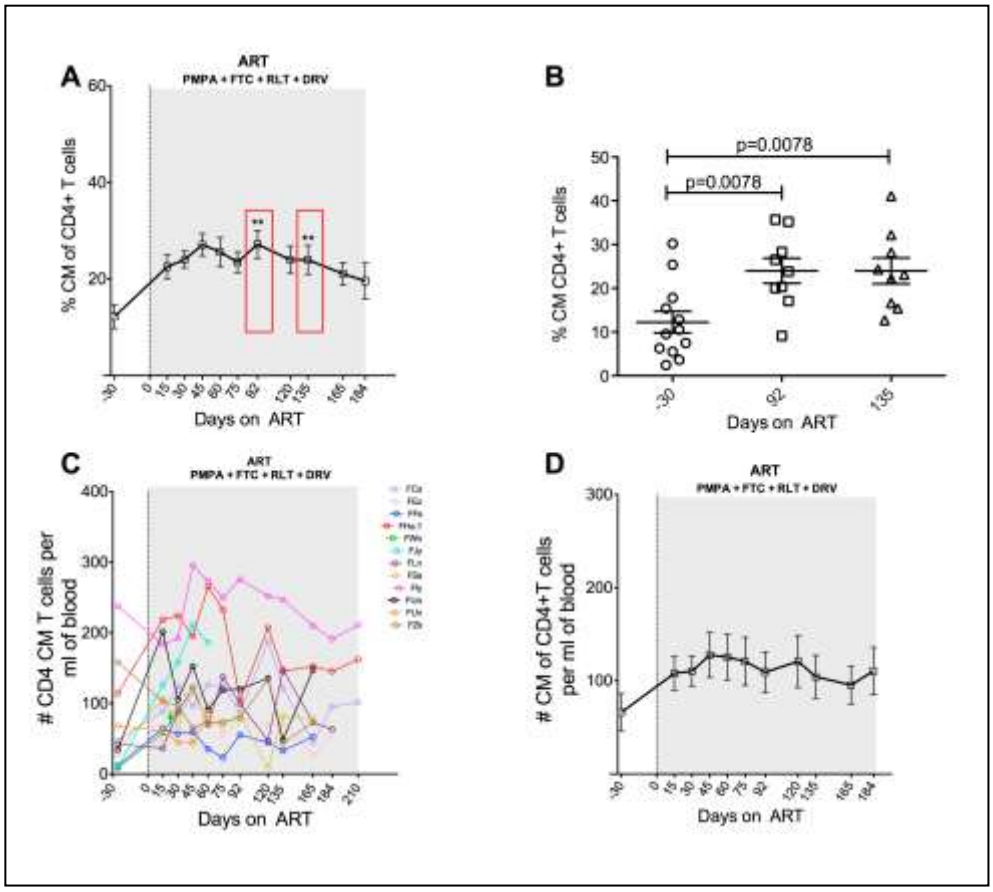

Figure 22. CD4+ T cells TCM dynamics in cART-treated SM. (A) Average of central memory (CM) frequency and their reduction (B) after ART initiation. Mean values, standard deviations and statistically significant differences are shown (p<0.05, ** p<0.01 and *** p<0.001 vs baseline, day-30) (B) Absolute count trend (C) and average (D) for TCM subset in whole blood for the treated SMs.*

4.4.3.3 CD4+ Transitional memory

Chomont et al. have demonstrated that in human TTM together with TCM represent the major reservoirs of latent HIV-1 [225]. TTM cells showed to be particularly responsive to some cytokines, such as interleukin (IL)-7 in human infection. IL-7 usually drives homeostatic proliferation of all memory T cell subsets, thus may be responsible for promoting the expansion of the latently infected cells, specfically TTM [257].

Moreover, consistent with Chomont in 2009 described, our immunophenotypic evaluation of SMs TTM revealed that this subset presents an intermediate phenotype between those of TCM and TEM. CD4+ TTM cells express higher lever of CCR5, HLADR and PD1 than TCM and lower level of the same surface markers when compared with TEM [225].

Analysis of CD4+ T dynamics showed that cART does not exert appreciable effects on transitional memory T cells kinetics in treated animals (Figure 23). TTM were maintained during the course of cART, based on our assessed parameters, frequency (Figure 23 A-B) or absolute count (Figure 23 C-D).

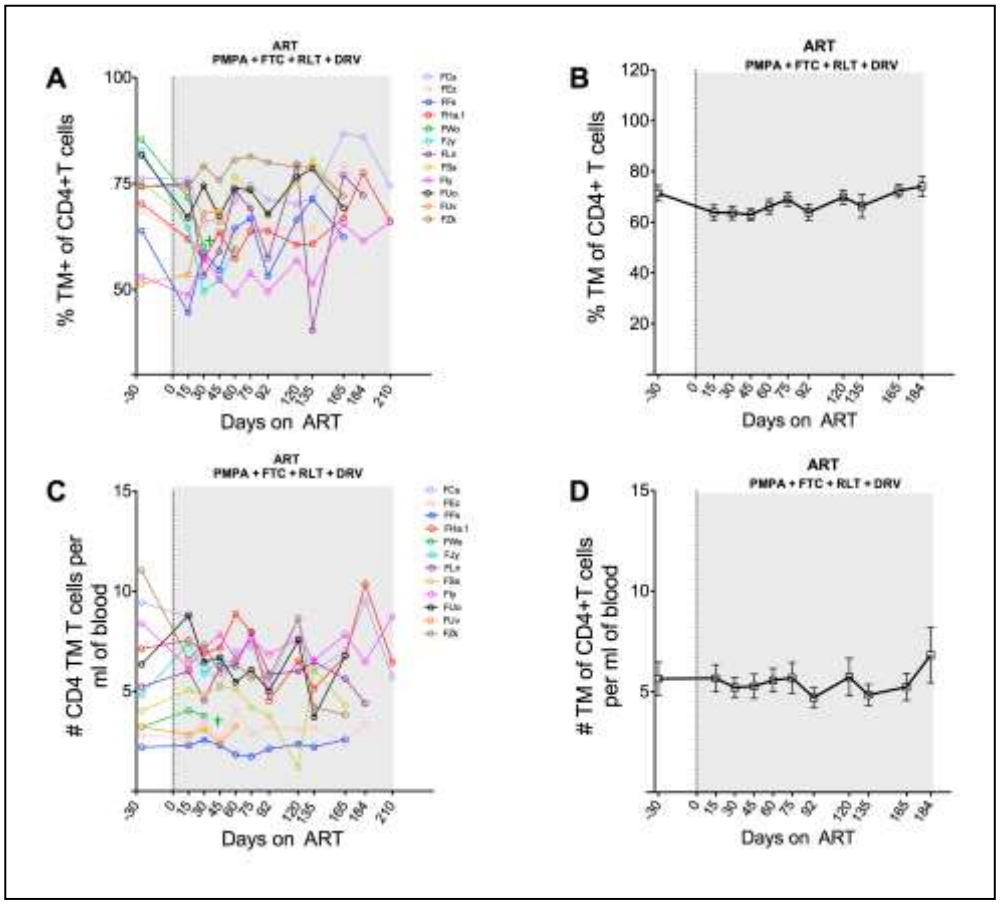

Figure 23. Maintenance of Transitional CD4+ T (TTM) cells subpopulation during cART. Definition of transitional memory T cells includes CCR7 and CD62L markers (shown before). (A) Frequency (left panel) and average (B) of frequency (right panel) of TTM (CCR7+CD62L-) in treated SMs (C) Absolute count (left panel) and mean values (D), standard deviations (right panel) are shown.

4.4.3.4 Memory stem CD4+ T cells

The memory T cell compartment is heterogeneous and the described conventional populations were represented by central memory, responsible for seeding the memory pool, and the effector memory that exhibit a more

differentiated phenotype. Initial works in mouse models, revealed the presence of a population of memory T cells that exhibits ‘stem cell-like’ features [258]. These stem cells, designated T memory stem cells (TSCM), present a naïve-like phenotype, expressing CD44low, CD62Lhigh markers; co-express canonical stem cells markers, such as stem cell antigen-1 (Sca-1), antiapoptotic molecule B lymphoma 2 (Bcl-2), IL-2Rbeta receptor and the chemokine receptor CXCR3 [258].

Later, Roeder et Al. identified phenotypically and functionally a similar population of long-lived memory T cell subset with stem cell-like properties. It has been found that TSCM are defined, in humans, as CD45RA+CD45RO-CD62L+CCR7+CD28+CD127+CD95+CD122+.

A homologous subset in RMs and pig tailed macaques was identified as CD45RA+CCR7+CD27+CD28+CD127+CD95+ [259], with intermediate expression of CXCR3, Bcl-2 and LFA-1 and suggestive of a population that is medial to both naïve and central memory [251, 258].

These cells have the unique ability to self-renew as well as a sustained proliferative potential allowing them to differentiate into all other memory T cell subsets, TCM, TEM and TTM. NHPs studies had given insights of TSCM distribution in tissues. CD4+ TSCM subset is mainly localized in peripheral blood and secondary lymphoid tissues, but is scarce in mucosal tissues, with a simililar tropism of naïve T cells. Both CD4 + and CD8+ TSCM have been identified in humans and NHP models. In the context of SIV infection, TSCM CD8+ T cells seem to be involved in the long-term maintainance of virus-specific CD8+ T cells-mediated responses.

While, despite the functional and phenotypic understanding of CD4+ TSCM, their contribution in HIV/SIV infection is unclear. Recently, Lichterfeld and his colleagues proposed CD4+ TSCM as the main memory subset that promotes long-term viral persistence in cART-treated patients [226].

As we approached other memory subsets, we aimed to better understand the role of CD4 + T memory stem cells (TSCM, CD45RA+, CCR7+, CD27+, CD28+, CD95+) in maintaince of viral reservoirs during cART treatment in non pathogenic SIV infection. We defined CD4+TSCM as previously Roeder described [260] and as represented in the figure below (Figure 24). Further inclusion of surface CD122 expression, confirmed we consistently evaluated TSCM based on the parameters recently described (data not shown, [251]).

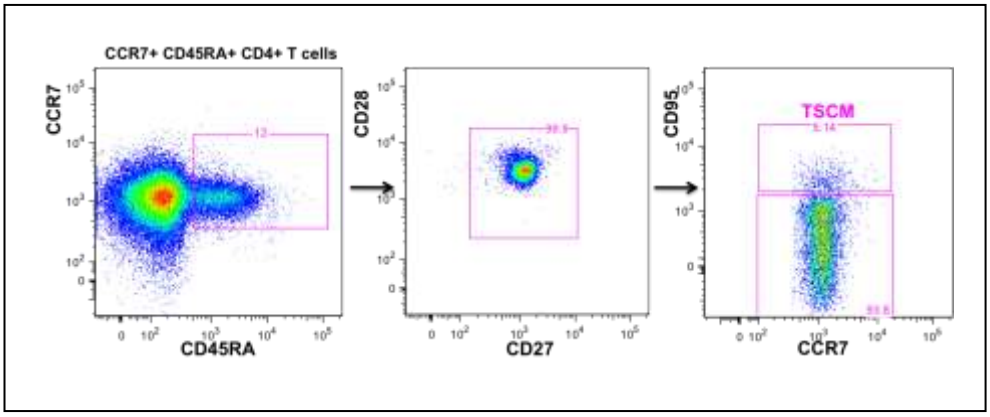

Figure 24. Phenotypic characterization of CD4+ memory stem T cells (TSCM). Definition of TSCM (highlighted in pink) based on the expression of surface markers CCR7, CD45RA, CD28, CD27 and CD95 on CD4+ T cells in one sooty mangabey.

The presence of such a pool of long-lived T cells with a less differentiated phenotype ensure a lifelong supply of protective immunity. During cART-treatment, TSCM frequency was maintained, with no significant fluctuations (Fgure 25 A). However, a significant expansion in TSCM counts was observed at day 60 ($p=0.0068$) (Figure 25 B-C). Interestly, given the ability of TSCM to differentiate into other memory, this may account for the increase in TCM in later time points during cART, as previously shown (Figure 22). Thus, this is a proof of principle that if TSCM are less infected

in natural hosts, may not contribute to the maintaince of viral reservoirs. TSCM may rather propagate and supply the memory niches with SIV-cleared new progeny.

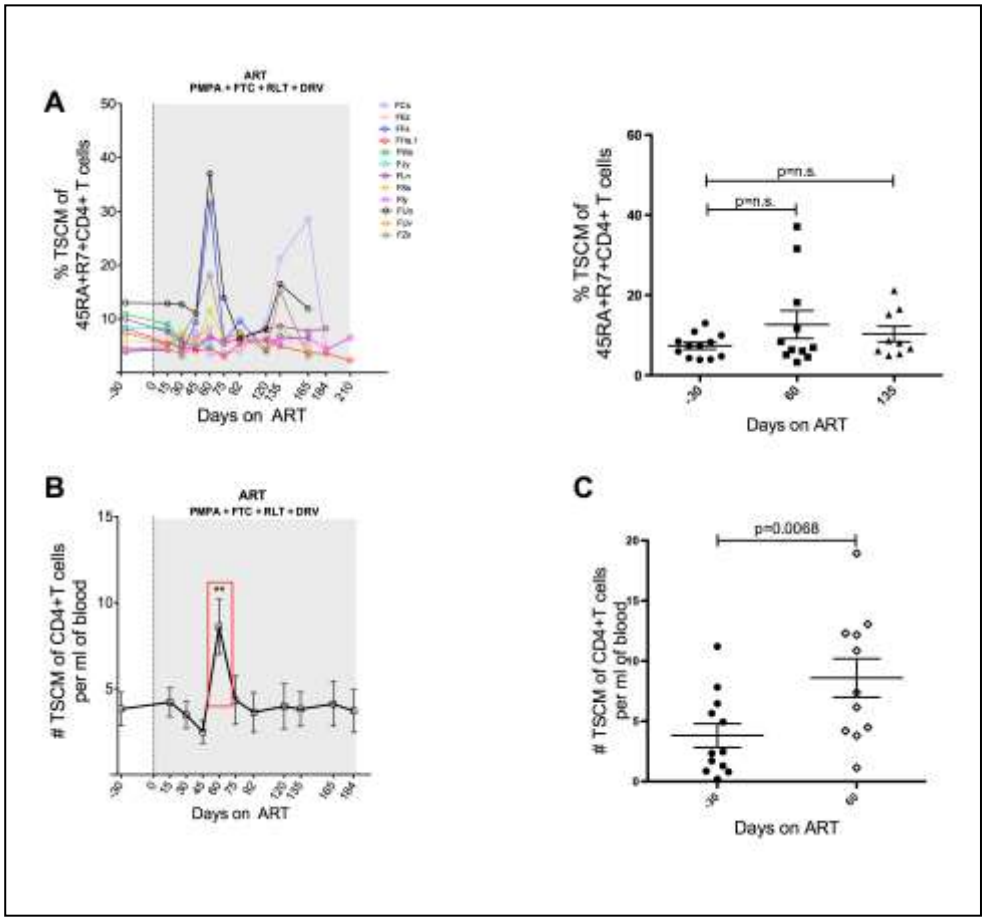

Figure 25. cART does not exert a net effect on CD4+ TSCM cells during antiretroviral therapy. (A) TSCM Frequency and absolute count (B) in the treated SMs. (C) Significant expansion of TSCM at day 60. Mean values, standard deviations deviations and statistically significant differences are shown (p<0.05, ** p<0.01 and *** p<0.001 vs baseline, day-30) are shown.*

4.4.4 cART impact in peripheral blood and rectal mucosal lymphocytes activation and proliferation

Systemic chronic immune activation is considered today, as the driving force that causes a progressive depletion of CD4+ T cells in pathogenic HIV/SIV infection and leads to significant increase of *in vivo* HLA-DR and CD38 expression on CD8+ T cells [256]. The limited immune activation of SIV-infected sooty mangabeys represents a marked difference with the generalized chronic immune activation in pathogenic HIV and SIV infections.

To further characterize the impact of cART on the levels of immune activation, we longitudinally assessed the expression of activation marker, HLADR on CD4+ and CD8+ T cell compartments. As expected, SMs presented low level of CD4+ T cells immune activation. HLADR expression remains stable during antiretroviral therapy in peripheral blood (Figure 26 A-B) as levels comparable to the baseline. Wheares, CD8+ T cells revealed a significant reduction in immune activation at day 165 when compared to day 75 ($p= 0.0273$) and at day 165 versus cART initiation day (day -30) ($p= 0.0039$) in systemic compartment (Figure 26 C-D).

Our observations of peripheral blood CD8+ and CD4+ T cells, mirrored the results found in the mucosae. Although, HLADR+CD4+ T cells fraction showed no major fluctuations during cART (Figure 27 A-B), the CD8+ T cells expressing HLADR were significally reduced at days 92 and 135 ($p= 0.0322$ and $p= 0.0195$) after cART initiation in mucosal compartment (Figure 27 C-D).

In HIV-infected individuals on cART, lower viral replication is associated not only with a reduced expression of T-cell activation markers, but also with a rapid decrease in T-cell turnover.

To assess this phenomenon in our model, we used variantions in Ki-67 expression as correlate of T cell turnover in our group of treated SMs. It has been demonstrated that SMs harbor low levels of CD4+ and CD8+ Ki67

expressing cells in peripheral blood versus those observed in RMs, where this exacerbated process promotes pathogenic progression [261].

Consistent with these data, we observed relatively minor cART induced-changes on frequency of proliferating CD4+ and CD8+ T cells both in systemic (average of Ki-67+CD4+ ranging between, 1.61 and 1.81%; average of Ki-67+ CD8+, 0.871 and 1.06%) and tissue compartments (average of Ki-67+CD4+ ranging between 2.19 and 2.56%; average of Ki-67+ CD8+, 2.013 and 3.8 %) (data not shown).

Consequences of elevated immune activation may contribute to viral persistence via increase of infectious targets, increase in viral production and upregulation of cell exhaustion marker PD-1 [262]. Antigen specific CD4+ and CD8+ T cells exhibit functional exhaustion *in vitro* when high levels of PD-1 are expressed, suggesting that PD-1 is a significant target molecule to restore HIV-response [263]. This has been evidenced in many clinical studies, in which HAART therapy dramatically reduced PD-1 expression on both CD4+ and CD8+ T cell compartments [263]. However, the effect of cART therapy in the non-pahthogenic model has yet to be understood. For this purpose, we evaluated PD-1 marker variantions in our cohort of SMs. According to our observations, no significant changes in the high level of PD1 expression were determinated in response to cART (data not shown).

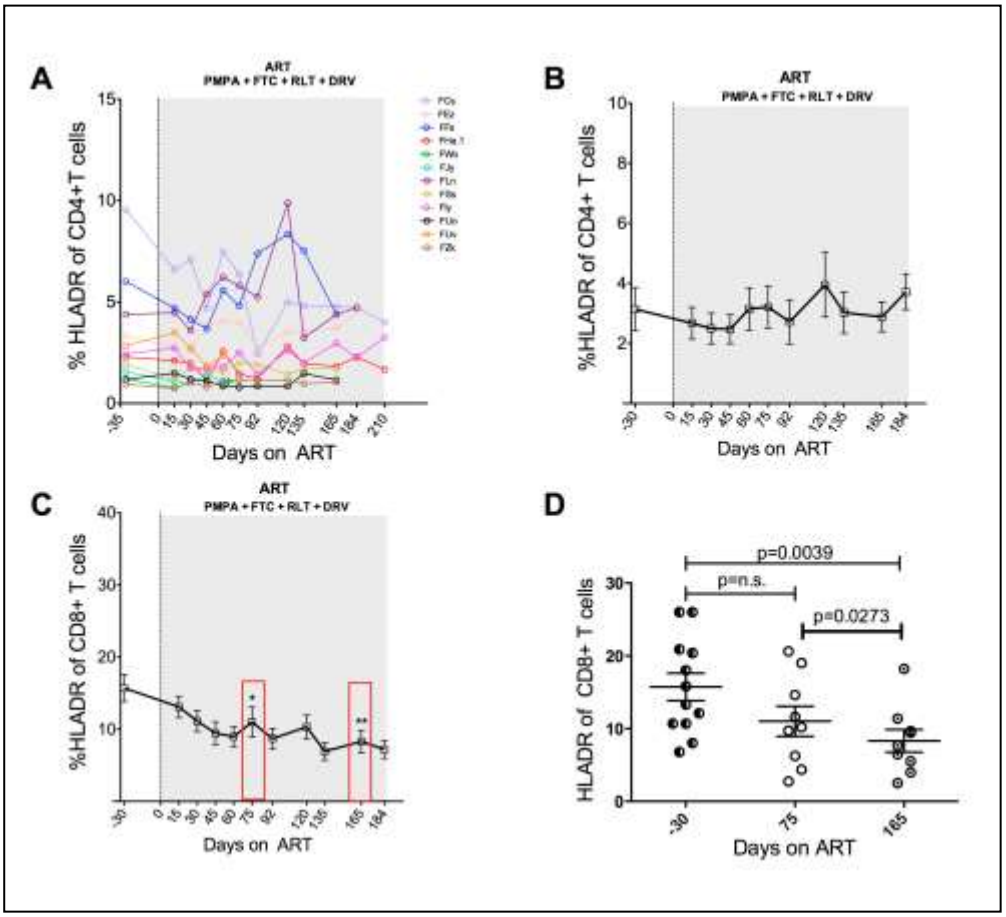

Figure 26. Kinetic of HLADR expressing CD4+ and CD8+ T cells in peripheral blood compartment. (A) Longitudinal analysis and average (B) of HLADR on CD4 + T cells before, during (shaded), and after cART. (C) Average and significant decrease (D) in CD8+ T cells expressing HLADR during cART. P values were determined by the Wilcoxon matched-pairs signed rank test. Statistic significant differences are shown (p<0.05, ** p<0.01 and *** p<0.001 vs baseline, day-30)*

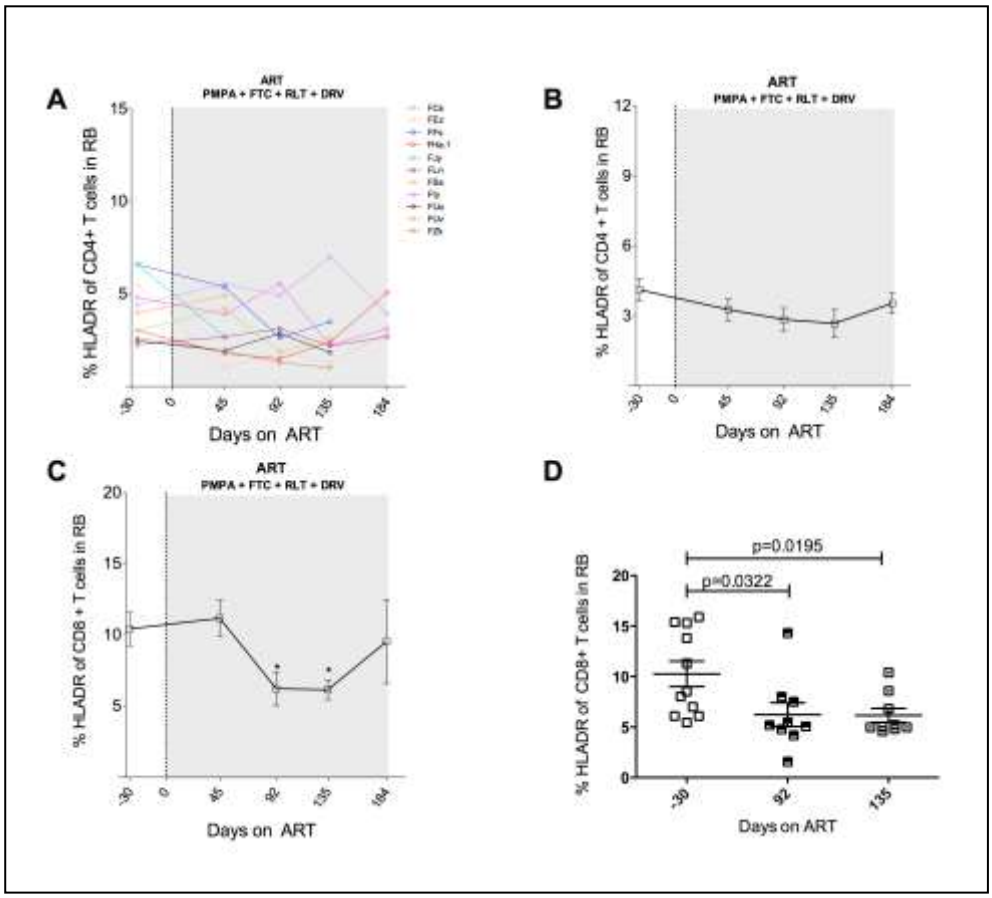

Figure 27. Kinetic of activated CD4+ and CD8+ T cells in rectal mucosae. General trend and mean values, standard deviations of HLADR+ on total CD4+ (A –B, top panel) and CD8+ (C-D, bottom panel) every six weeks from ART initiation. Statistically significant differences are shown (∗ p<0.05, ∗∗ p<0.01 and ∗∗∗ p<0.001 vs baseline, day-30). P values were determined by the Wilcoxon matched-pairs signed rank test.

4.4.4.1 Immunophenotypic assessment of CD4+T cell compartment during course of cART

To better elucidate the role of viral replication in SIV-persistence, we interrogated CD4+ T cell subsests proliferative and activation status and if it may contribute to SIV reservoirs. For this purpose, we measured proliferative capacity, considering expression of Ki67, activation levels, using HLADR expression and finally phenotypic exhaustion was determined by PD1 marker, on CD4+ TCM, TTM, TSCM and TEM during the course of cART.

cART had little influence on HLADR expression on CD4+ TSCM, TCM and TM fractions in all animals (Figure 28 A). However, a cART-induced increase of activated TEM at day 184 ($p= 0.0103$) in comparison to pre-cART, was observed (Figure 28 B). Possibly the inhibition of replication mediated by cART contributed to new functional TEM with effector phenotype.

Moreover, Ki67+ TEM cells (range of Ki67+ cells, 3.6–3.8%) were present in higher frequency. In contrast, TCM cells and TTM cells were characterized by low to intermediate levels of Ki67 expression, as indication of potential sources of SIV viral reservoir. Interestingly, consistent to previous reports [251], CD4+ TSCM from treated SMs showed higher levels of Ki-67 (Figure 28 C), suggesting that this smaller pool of cells may maintain CD4+ memory T cell homeostasis through higher basal rates of proliferation (Figure 28 C).

Finally, our results indicated that PD-1 expression remained upregulated at higher levels particularly in the late differentiated CD4 subsets (TEM cells). While, CD4+ TTM and TSCM cells exhibited higher frequency of PD-1+ expression than TCM cells, but lower than TEM cells (Figure 28 D).

Taken together these results suggested that SIV may potentially persist in TCM and TTM cells by continuous low-level of proliferation, lower expression levels of co-inhibitory molecule PD-1 and less activated

phenotype, ensuring the persistence of integrated viral DNA through mitosis.

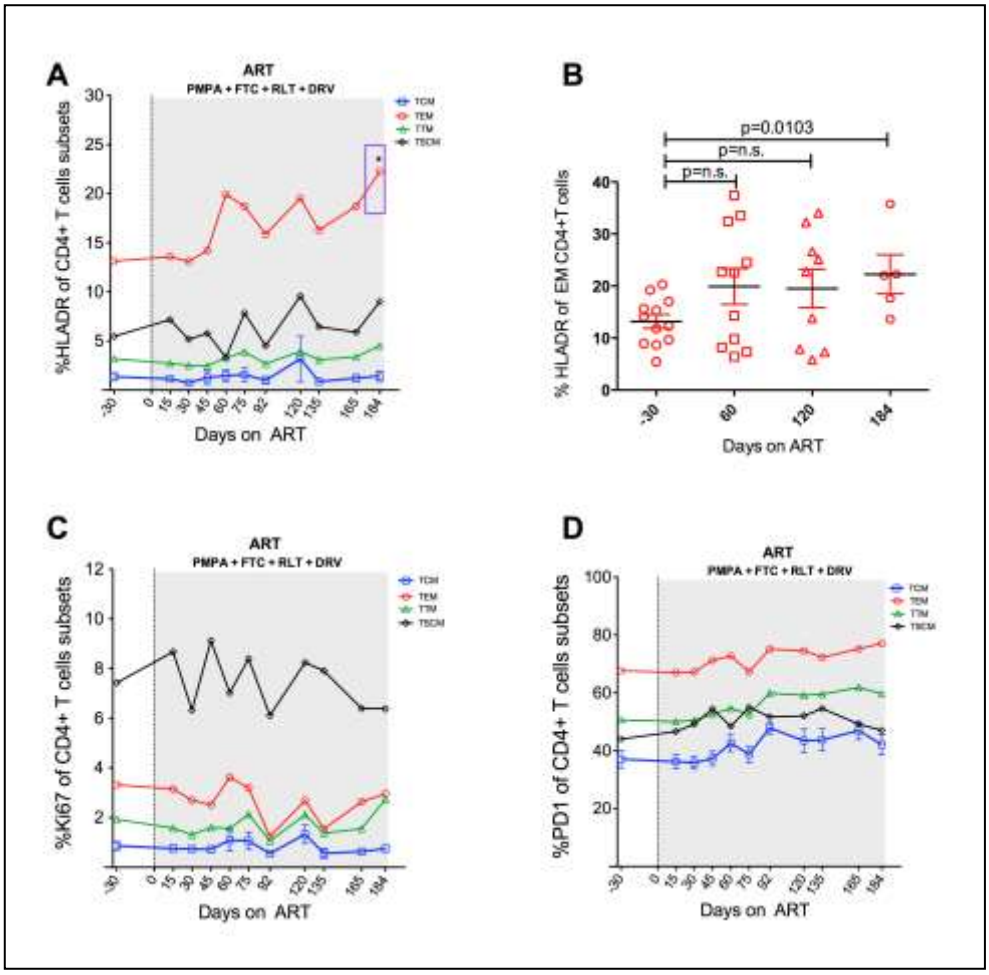

Figure 28. Variations in activation, proliferation and exhaustion expression levels in CD4+ T cell population. Frequency of CD4 expressing HLADR (A-B), Ki67 (C) and PD-1 (D). Mean values are shown as horizontal bars and standard deviations. Statistical significance was determined using the Wilcoxon matched-pairs signed rank test (p<0.05, ** p<0.01 and *** p<0.001 vs baseline, day-30)*

4.5 Reduced SIV infection of SMs CD4+ T cell subsets after ART initiation

The most significant barrier to HIV cure is the establishment of stable viral reservoirs in the early phases of infection. Different studies have evaluated the impact of early initiation of ART on viral reservoirs in both HIV-infected patient and SIV-infected RMs. However, as documented in a recent study, even RMs challenged intrarectally with SIVmac251 and receiving early ART on day 3, experienced viral rebound after discontinuation of ART following 24 weeks of SIV fully suppression [247]. It is becoming more clear tha viral reservoirs are seeded rapidly after HIV/SIV infection, even before post-infection peak of viral replication is observed in plasma. Moreover, previous findings in monkeys indicated that the size of the SIV-reservoir in PBMCs and tissues increases significantly earlier after SIV infection, around day 7 and day 10 [246].

Despite current treatments induce a rapid decrease in plasma viremia, SIV/HIV-infected cells can persist in various compartments, such as blood and immunologically privileged sites, central nervous system, gut mucosa, lymph nodes and male and female genital tract [217, 222, 223], due to ongoing viral replication in these sites.

A deep characterizations of the main component of viral reservoirs has revealed latently infected are represented by resting CD4+ T cells carrying an integrated copy of viral DNA [217, 219, 220, 221]. Indeed, accordings to recent findings, viral reservoir resulted to be largely made up of CD4 memory T-cells, including central, transitional and effector memory cells in humans and in RMs [225, 264]. Recently, Maria Buzon et Al., identified a pool of long-lasting cells, memory T cells stem cells that harbor high levels of HIV DNA despite long-term antiretroviral therapy in HIV-infected individuals [226].

In NHPs models, remarkably differences in the pattern of infection of CD4+ T cell subsets during natural and non-natural SIV infection of sooty

mangabeys and rhesus macaques, has deeply been described. In 2011, Paiardini et al., showed that, in contrast to the pathogenic NHP counterparts, SMs CD4+ TCM cells fraction expresses low CCR5 levels. CD4+ TCM relatively protection from virus-mediated depletion, preserves CD4+ homeostasis and promotes an AIDS-free status in this species [112]. In addition, a recent published study revealed that direct virus infection of CD4+ TSCM is a peculiar feature of SIV-infected RMs, with the majority of SIV-infected SMs lacking SIV DNA within the same subset [251].

In the light of these results, since specific SMs memory CD4+ T cell subsets resulted to be particularly resistant to SIV infection, we wanted to test *in vivo* the relatively contribution of specific infected cell populations to virus persistence in cART-treated SIV-infected SMs.

For this purpose, we assessed the analysis of the memory CD4+ T cell compartments, sorting CD4+ T cell subsets from all SMs before and during cART. On the basis of surface expression of CD45RA, CC chemokine receptor-7 (CCR7), CD28 and CD95, we identified the different CD4+ T cell subsets, including effector memory (EM), central memory (CM), trasitional memory (TM) and memory stem cells (TSCM). Moreover, TTM, TEM and TCM were further distinguished based on CD62L expression. Highly sensitive quantification of total SIV proviral DNA on the sorted cells was then performed. The number of gag DNA copies amplified from each sample was normalized to the number of cells in each PCR, measured as expression of albumin gene.

The investigation of the CD4+ TCM, TEM, TTM and TSCM fractions highlighted their different susceptibility pattern to SIV infection (Figure 29).

Here we show cell-associated SIV-DNA data of 5 out of 12 SMs, including animals receiving cART for 2 and 6 months.

Results showed that before cART initiation, SIV-DNA content in TEM was higher than in TCM, confirming previous data [112]. Short-lived CD4+TEM,

as main source of viral replication, confirmed to represent the major SIV reservoirs contributor in absence of cART. At day 45, a rapid reduction in SIV-DNA amount was observed in all CD4+ T cells subsets of 5 cART-treated SMs (Figure 29). Moreover, our results revealed that TCM and TSCM showed limited contribution to the pool of latently SIV-infected cells, with lower levels of SIV-DNA if compared with TTM and TEM (Figure 30 A-B-C-D). Interestingly, one animal (FEz) exhibited undectable levels of gag SIV copies in TCM and TSCM fractions both prior to cART treatment and during the treatment, suggesting the peculiar absence of latentely SIV-infected long-lived cells harboring SIV-DNA in that animal.

Overall, our data confirmed the role of the memory compartment in SIV persistence. cART was effective in reducing the size of the viral reservoirs in all the CD4+ T cell subsets and specifically, in TEM cells. Consistently with previous data, TCM and TSCM resuted to be relatively resistant to SIV infection in natural host, suggesting that once SIV-infected TEM are cleared and cART interrupted, no viral rebound may be observed.

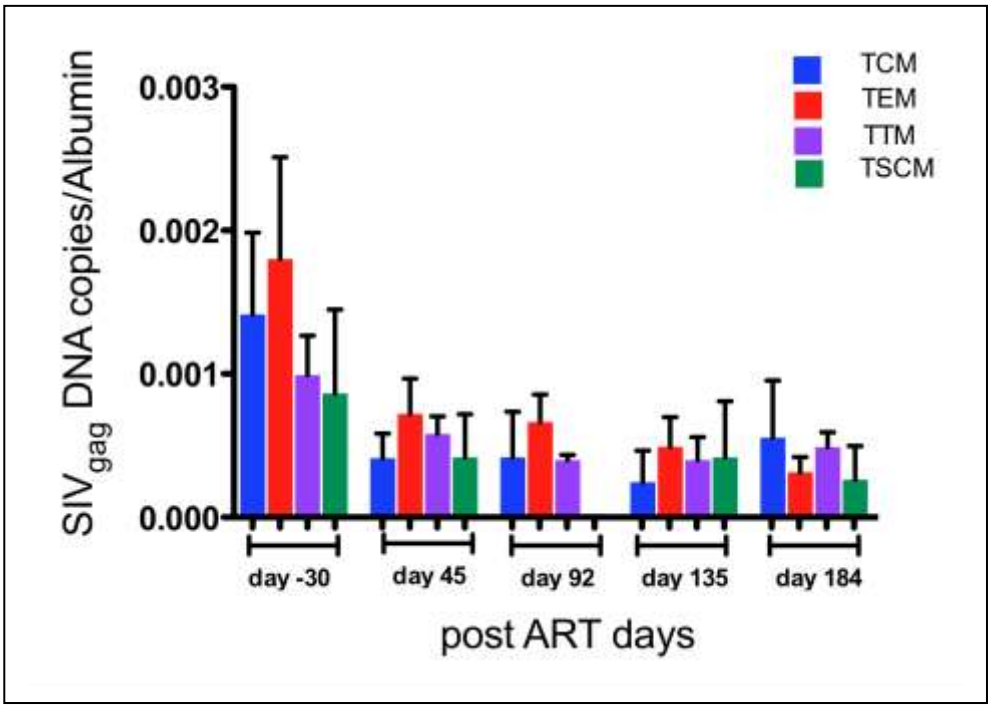

Figure 29. Effect of cART on SIV-DNA content of CD4+ T cell subsets. Contribution of TCM (blue), TEM (red), TTM (purple) and TSCM (green) CD4+ T cell subsets to the pool of SIV-infected cells was calculated in 5 SMs that interrupted cART. Total SIV DNA copy number was determined in sorted subsets by highly sensitive real-time PCR. In the graph, error bars represent s.e.m.

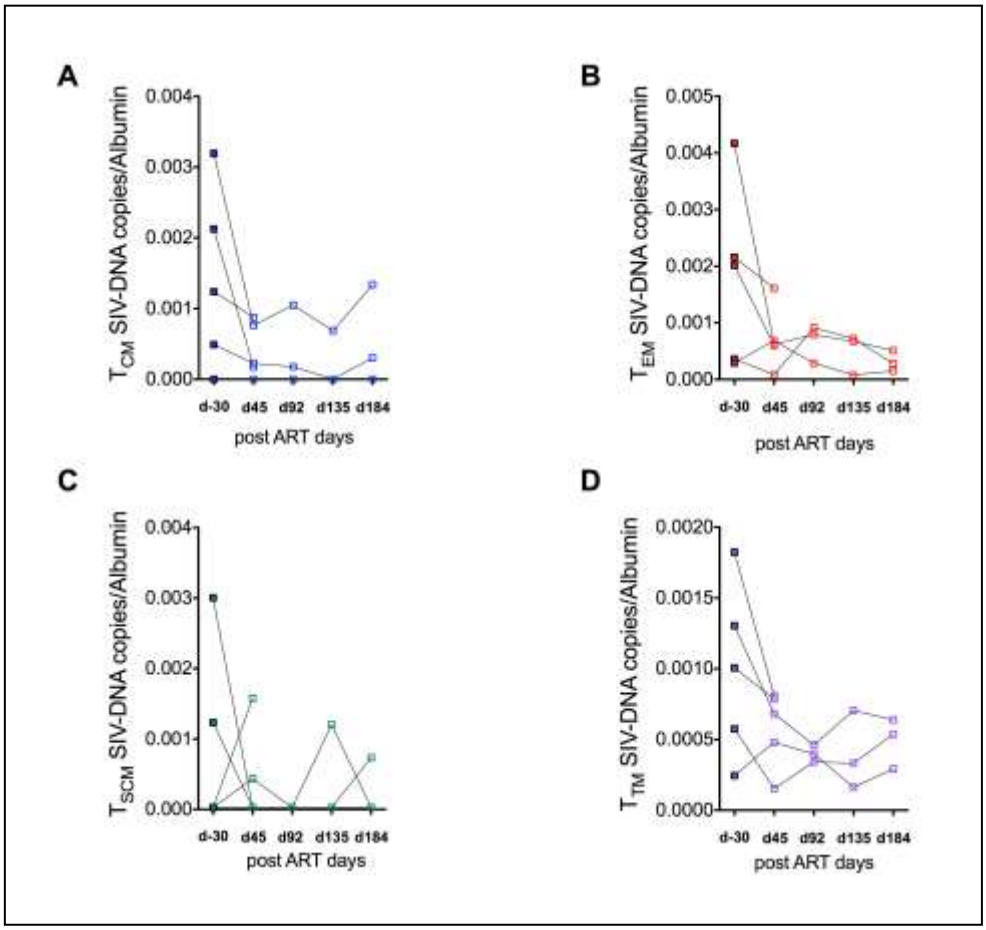

Figure 30. CD4+ T cell subsets harboring integrated SIV-DNA. Analysis of TCM (A), TEM (B), TSCM (C) and TTM (D) prior to cART initiation, day -30 (full squares), and during cART, day 45, 92, 135 and 184 (empty squares). Results were expressed as the SIV copy number was calculated to the albumin housekeeping gene.

4.6 Virological features of ART-interrupted SMs

HIV-1 patients on ART show a dramatic and rapid decrease in plasma viremia below the limit of detection, reflecting the clearance of free HIV virions and productively infected CD4+ T cells. However, although cART is

very effective at containing HIV-1 spread within the body, viral load can reboundas upon interruption of therapy. The main cause of viral rebound is the presence of earlier established stable reservoirs that promote viral replication.

The focal of our work is to understand the mechanisms of the complex virus-host interactions that lead to persistent infection. By intensive study and implementing fully suppressed SIV replication with a potent new combination of antiretroviral therapies, we had the possibility to study, in depth, the sources of residual virus and kinetics of short-lived CD4+ T cells. We successfully observed the inhibition of SIV replication during cART, indicating the combination of antiretroviral drugs used resulted to be safe, well tolerated and efficient in viral replication suppression. We also assessed which is the main CD4+ T subsets involved in SIV persistence in a non-pathogenic model such as SMs, revealing that TEM and TTM were the major contributors to SIV-reservoirs.

We aimed to further interrogate the effect of cART in SIV-reservoirs after cART interruption. For this purpose, treatment was administrated for different temporal periods, to understand the best timing to achieve full SIV-viremia suppression and elimination of residual viral replication. According to our experimental study design, the selected SMs were further divided into four treatment-interruption groups. Specifically animals received cART for 2, 6, 9 and 12 months. Plasma viral load was measured in the animals after cART suspension at different time points.

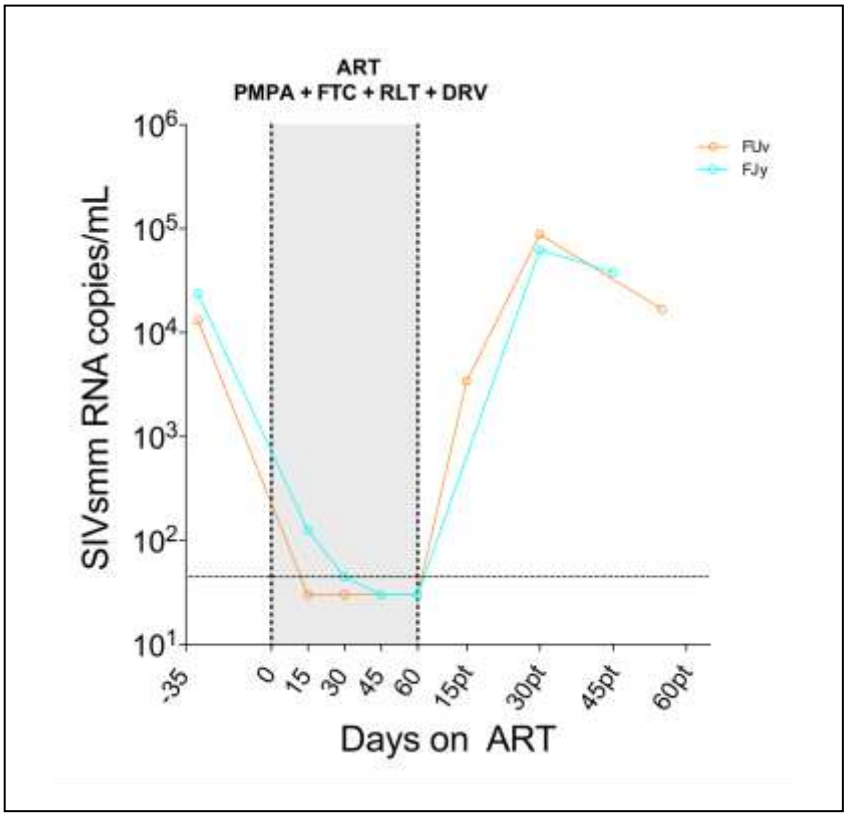

Figure 31. Viral kinetic in 2 months of cART group. SIV-RNA levels in two SIV-infected SMs, FJy and FUv, that received antiretroviral therapy from day 0 to day 60. Gray shading depicts periods on cART.

We obtained full SIV-suppression in presence of cART in the treated-SMs. Animals FJy and FUv interrupted cART at day 60. As expected, the stoppage of therapy at two months resulted in a rapid rebound in SIV viremia. The viral kinetic was remarkably similar to those observed during acute SIV infection of SMs [111, 252] (Figure 31). Animals experienced a high peak at day 30 with a viral set point around 10^4 SIVsmm RNA copies/ml at latest time point.

Viral kinetics after stoppage of cART 6 months later (day 184) revealed that animals FLn and FEz experienced an initial peak at earlier time point

comparable to the baseline levels (Figure 32). Interestingly, reduction of viremia was observed at day 30 post cART interruption. As confirmed to our previous data, FEz particularly experienced a dramatic decay in viremia close to undetectable levels. Thus, data suggested that 6 months cART treatment was effective in reducing the source of latent viral burden and that possibly SMs' immune system was able to control virus replication, promoting the elimination of the remaing SIV-infected cells.

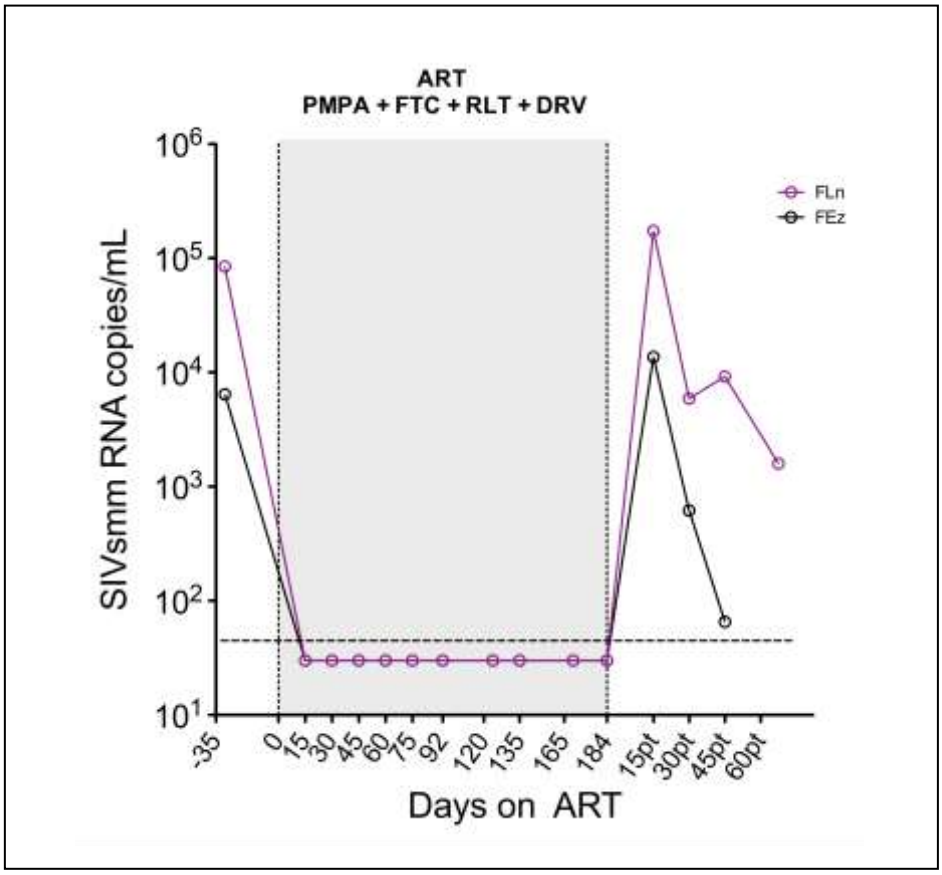

Figure 32. Viral kinetics following cessation of cART after 6 months. SIV-RNA levels in two SIV-infected SMs, FLn and FEz, that received antiretroviral therapy from day 0 to day 184. Gray shading depicts periods on cART.

4.7 Immunological variations in SMs following cART cessation

In this study, we wanted to examine quantitative aspects of CD4+ and CD8+ T cells dynamics throughout the duration of the treatment but above all, after cART cessation, to better define the immunologic correlates outcome in SMs that received cART. Specifically, we show here an analysis of the all interrupted animals (2 and 6 months) at days 15 and 30 post cART.

4.7.1 Viral rebound and impact on CD4+ T cell population

Interestingly, interruption of therapy did not affect the percentage or absolute count of CD4+T cells in the studied animals (Figure 33 A-B). These data indicated that level of viral replication does not alter the magnitude of circulating CD4+T cells in natural host species.

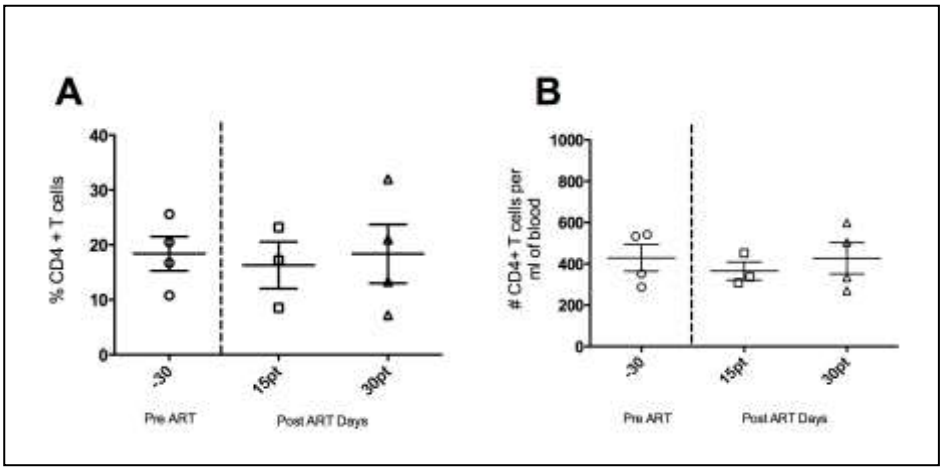

Figure 33. Effect of cART interruption on circulating CD4 + T cells. Frequencies and counts of CD4 + T cells are not decreased following cART interruption. (A) Frequency and (B) absolute count mean values of CD3+CD4+T cells fraction in peripheral blood prior to (day -30) and at cART interruption (dotted line). Data of days 15 and 30 post cART in all interrupted SMs are shown.

4.7.2 CD8+ T cells and the control of plasma viremia in 6 months group

The rapid and initial viremia peak at day 15 post cART, followed by the control of rebound viremia to pre-cART set point levels and even lower in animals on cART for 6 months, brought us to investigate a possible involment of CD8+ T cells.

Interestingly, no changes in CD8+ T cell frequency or counts were observed during the course of the study (data not shown) and early following cART stoppage in the animals that received cART for 2 and 6 months (Figure 34 A-B). No remarkable expansion in CD8+ T lymphocytes was observed, so likely the reduction of viremia may be the result of cART effect on viral reservoirs size.

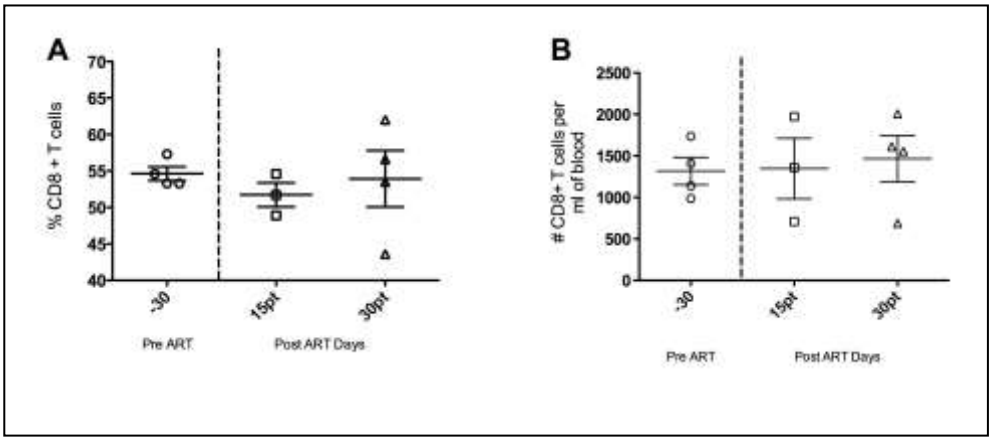

Figure 34. Interruption of cART and variantions in CD8 compartment. (A) Frequency and (B) absolute count mean values of CD8+T cells per mm^3 of blood. CD8+ T cells compartment was monitored throughout treatment and after cessation periods (dotted line).

4.7.3 Immunoactivation and proliferation levels on lymphocytes after cART interruption

Coincident with the increase of viral replication upon the termination of cART, a modest increase in the activated CD4+ and CD8+ T cells was observed at days 15 and 30 post cART interruption (Figure 35 A-B).

Interestingly, we observed an augmentation of Ki67+CD4+ T cells at days 15 and 30 post cART versus pre-cART time points ($p= 0.0035$ and 0.0321, respectively). It was unclear if initial SIV replication-peak induced proliferation of SIV-specific CD4+ T cells or, alternatively, if it's a result of increased immune activation levels on lymphocytes after cART interruption, that contributed to the transient virus replication higher than the pretherapy set point [110] (Figure 35 C). Moreover, in our SMs, we observed that cART interruption induced an increase in the level of proliferating CD8+ T cells (Figure 35 D), event that could be a self-limiting response to the increased viral burden in the context of cART interruption [110].

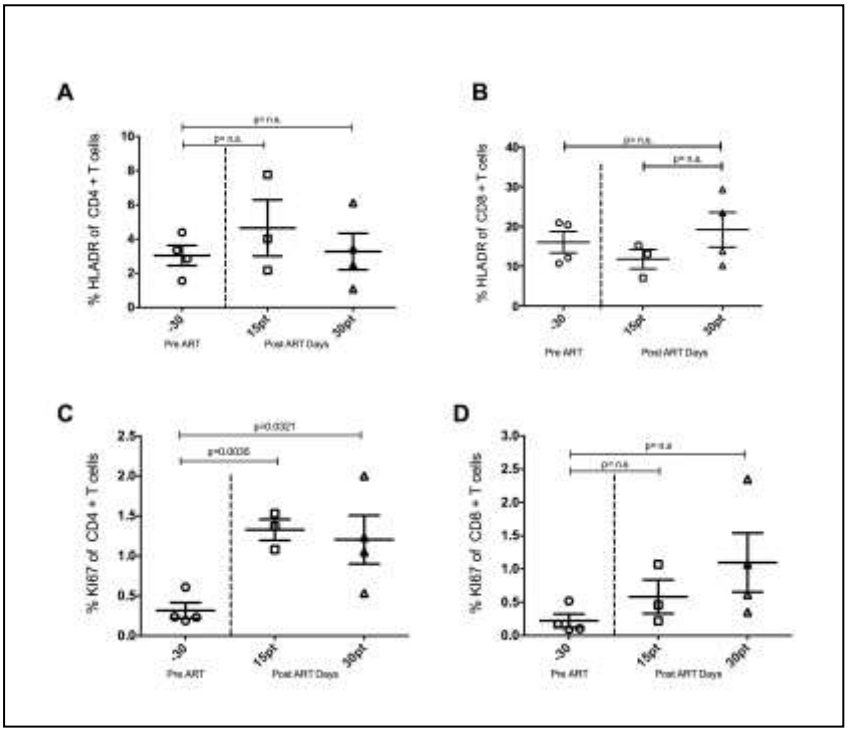

Figure 35. Immunological changes following cART cessation on CD4 and CD8 activation, proliferation status. Frequency of HLADR expressing CD4+ (A) and CD8+ (B) T cells in peripheral blood. Ki67 expression was measured on CD4+ (C) cells and CD8+ T cells (D) after cART cessation periods. Statistical significance was determined using the Wilcoxon matched-pairs signed rank test (∗ p<0.05, ∗∗ p<0.01 and ∗∗∗ p<0.001 vs baseline, day-30)

Conclusions and Future Directions

5. CONCLUSIONS AND FUTURE DIRECTIONS

The development of highly active antiretroviral therapy (HAART) has dramatically improved the clinical outcome in HIV-infected, increasing their life expectancy. Nowadays, a large number of very potent antiretroviral drugs have dramatically reduced HIV-mortality and morbidity. However, HIV eradication remains unachievable, due to the presence of persistent viral reservoirs in peripheral blood and tissues of infected patients [217, 219, 220-223]. Consequently, a thrust of HIV research has been to develop therapeutic strategies that can boost host immunity to control viral replication upon discontinuation of cART or eliminate persistent viral reservoirs. However, our knowledge of the systemic cellular nature of HIV reservoirs is incomplete. To provide elucidations on the possible mechanisms that rule ongoing SIV-residual replication, we performed an intensive examination of latently infected cellular components of SIV-reservoirs in a non-pathogenic model of SIV infection undergoing cART. For this purpose, twelve chronically SIV-infected SMs were treated for 2, 6, 9 and 12 months with combination antiretroviral therapy (cART) consisting of four drugs (PMPA/Tenofovir, FTC/Emtricitabine, Raltegravir and Darunavir). Of particular interest in our study, the achievement of a full suppression model of viral replication in SIV-infected SMs by the use of a novel and powerful cART regimen. Moreover, our focus was to investigate the cellular composition of SIV-viral reservoirs in blood and mucosal compartments and cART impact on productively infected cells in non-pathogenic infection.

Here, we proved that the four-drug regimen administrated to all animals, resulted to be safe, well tolerated and with no discernible side effects. cART-treated SMs, experimentally infected with SIV, experienced a rapid decline from 2- to 3-log decrease- of plasma viremia at the earliest time points, suggesting that cART resulted to be sufficiently effective in the

inhibition of SIV replication with plasma viral load below the limit of detection. In most HIV-infected individuals, the viral suppression replication achieved by highly active ART administration results in an increase in CD4+T-cell counts as well as a decrease in the levels of immune activation. In this cohort of SMs, we observed only minor fluctuactions in CD4+ T cell number in systemic compartment, while a marked reconstitution of mucosal CD4+ T cells at the time of cART-induced suppression of viral replication. With respect to level of immune activation, although we observed that cART induced minor decrease in T-cell proliferation (measured by Ki67 expression) and immune activation (measured by HLADR expression) on CD4+T cell, activated and proliferating CD8+ T cells were remarkably reduced in blood and mucosa of treated SMs.

Several studies strongly revealed that HIV latency is mainly due to resting long-lived CD4+ T cell in memory pool, that persist in HIV-infected individuals on cART. We showed that the content of SIV-DNA in infected CD4+ T cells resulted to be reduced during suppressed cART in SIV-infected SMs. Consistent to previous studies [112, 251], SMs were particularly susceptible to cART, due to lower infection levels in specific CD4+ memory subsets. Specifically, SMs CD4+TCM and TSCM revealed a relatively resistance to SIV during cART treatment. Moreover, the evaluation of cART impact on the dynamics of CD4+ T cell pool, revealed redistribution in the different subsets. In particular, a rapid CD4+ TEM reduction was observed during cART administration. Interestingly, we described an expansion of low SIV-DNA infected CD4+ TSCM and TCM after cART initiation, that fuel, according to our hypothesis, the re-population of memory niches with nascent progeny, harboring cells with reduced or no SIV virus.

Moreover, the current study also gave us the opportunity to investigate the kinetics of viral replication and immunobiology in SMs after cessation of

antiretroviral therapy. When cART was interrupted, although all animals preserved CD4+ and CD8+T cell populations at levels comparable to the pre-cART, activated and proliferating T cell in both pools were augmented.

Interestingly, SIV-infected SMs experienced a rapid rebound followed the interruption of cART, characterized by a peak of viral replication occurring within 2 weeks post cART interruption. However, interestingly, after 6 months of cART a reduction of latent viral burden was observed.

We are currently investigating cART-induced effects in the remaining cohort of SMs, receving treatment for 9 and 12 months. Our previous observations on 2 and 6 months interruption groups were very encouraging. The dramatic decay of viremia in presence of cART, the early redistribution of CD4 +T cell subsets and the viral kinetics after cART cessation, allow us to be confident to achieve a complete clearance of SIV-infected cell in viral reservoirs for prolonged cART periods.

In conclusion, the observed reduction of SIV-reservoirs size in the setting of a full SIV-suppressed NHPs model, provided crucial insights in the investigation of SIV/HIV latency mechanisms. Our results will offer a new experimental *in vivo* platform to address innovative interventions aimed at eradicating viral persistence in human HIV infection.

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Scientific products

7. SCIENTIFIC PRODUCTS

List of the scientific products (oral communication, poster presentation, manuscript)

POSTERS

- V. Rainone, D. Trabattoni, F. Penagini, F. Dinello, F. Calascibetta, G. V. Zuccotti, V. Giacomet, A. Viganó, M. Clerici. "*HPV vaccination in ARV-treated HIV-infected adolescents and young adults induces strong HPV-specific cell-mediated immune responses*", International Symposium HIV and Emerging Infectious Diseases 2014 - Marseille, France. 21-23 May 2014.

- V. Rainone, D. Trabattoni, F. Penagini, F. Dinello, F. Calascibetta, G. V. Zuccotti, V. Giacomet, A. Viganó, M. Clerici. "*HPV vaccination in ARV-treated HIV-infected adolescents and young adults induces strong HPV-specific cell-mediated immune responses*", 7th IAS Conference on HIV Pathogenesis, treatment and prevention – Kuala lumpur, Malaysia. 30 June – 03 July 2013.

- C. Mandò, C. De Palma, G.M. Anelli, M. Figus, M. Mazzocco, F. Calascibetta, D. Trabattoni, T. Stampalija, E. Ferrazzi, E. Clementi, I. Cetin. "*Increased trophoblast oxygen consumption in IUGR*", 60th Annual Meeting of the Society for Gynecologic investigation – Plasticity: Molecules to Motherhood and Beyond – Orlando, Florida, USA. March 20-23 2013.

- Bandera, D. Trabattoni, N. Squillace, A. Muscatello, F. Calascibetta, A. Maloberti, C. Giannattasio, V. Marcandalli, M. Clerici and A. Gori."*Early changes in adhesion molecules expression and endothelial function in*

patients initiating ART with Atazanavir or Lopinavir", 20th CROI 2013 – Conference on Retroviruses and Opportunistic Infections – Atlanta, Georgia, USA. March 3-6 2013.

ORAL COMUNICATION

- F. Calascibetta, V. Rainone, V. Temchura, K. Uberla, A. Clivio, M. Nebuloni, E. Lauri, D. Trabattoni, F. Veas, M. Clerici. "*Role of the Mucosae-Associated Epithelial Chemokine (MEC/CCL28) in the modulation of the immune response against viral infections*" presented at ICAR 2012 - Italian Conference on AIDS and Retroviruses – Napoli. 10-12 giugno 2012.

FUTURE SUBMISSIONS

- Abstract Submission within December 22nd 2014: "Keystone Symposia on Molecular and Cellular Biology", Boston, Massachusetts, USA. April 26—May 1, 2015.
- Manuscript: V. Rainone, V. Giacomet, F. Penagini, V. Fabiano, *F. Calascibetta*, C. Mameli, S. Pisanelli, G.V. Zuccotti, M. Clerici, D. Trabattoni, "HPV Vaccination Induces Strong HPV-Specific Cell-Mediated Immune Responses in HIV-infected Adolescents and Young Adults" submitted to the Journal Editor and waiting for review.
- Manuscript on work of the present thesis will be submitted on 2015.

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