Universidade do Minho
Escola de Ciências da Saúde

Ana Maria Lacerda Morgado
Fernandes de Carvalho de Aboim Horta

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Células T CD4^{+} reguladoras na reconstituição imune de indivíduos infetados por VIH

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Tese de Doutoramento em Medicina

Trabalho realizado sob a orientação de:
Prof. Doutora Margarida Correia-Neves
Professora Associada
Escola de Ciências da Saúde
Universidade do Minho, Braga, Portugal

Professor Doutor Rui Sarmento e Castro
Professor Catedrático Convidado
Escola de Ciências da Saúde
Universidade do Minho, Braga, Portugal

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Declaração

Nome: Ana Maria Lacerda Morgado Fernandes de Carvalho de Aboim Horta

Endereço Eletrónico: agaboim@hotmail.com

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Orientadores:

Prof. Doutora Margarida Correia-Neves

Professor Doutor Rui Sarmento e Castro

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Regulatory $CD4^+$ T Cells in the Immune Reconstitution of HIV-Infected Individuals

Abstract

Acquired Immunodeficiency Syndrome (AIDS) caused by the human immunodeficiency virus (HIV) is characterized by a steady imbalance in the immune system mainly at the level of the cell-mediated immunity, leading to a final stage of inability to counteract major life threatening infections and tumors. With the advent of highly active antiretroviral therapy (HAART), HIV infection became manageable as a chronic disease. However, HAART does not fully restore health and does not always lead to the recovery of the $CD4^+$ T cell numbers to normal levels.

Regulatory T cells (Treg), a specialized subset of T cells, play an essential role in the control of immune responses. In the context of HIV infection, Treg may be considered harmful by suppressing HIV-specific immune responses or beneficial, by dampening excessive immune activation. It has also been hypothesize that their deregulation could hamper immune recovery and be involved in the immune reconstitution inflammatory syndrome (IRIS). Despite many reports concerning the role of Treg in the immune system recovery of HIV-infected patients under HAART, the subject remains controversial. Clarifying Treg function in this process is of paramount importance to better understand the immune reconstitution in HIV-infected patients and therefore, potentially use this information to improve the disease management.

Focused on understanding the role of Treg in the immune system reconstitution, we endeavor a work involving exclusively HIV-infected patients under HAART. Two studies were performed: a cross-sectional study involving HIV-infected patients aviremic under HAART, and a longitudinal study involving HIV-infected patients HAART-naïve, who were followed since the initiation of therapy, through several time-points and until they have performed two years of HAART (providing they were adherent to HAART and the treatment was effective at decreasing the viral load).

We found that untreated HIV-infected patients in a more advanced stage of the disease (<200 $CD4^+$ T cells/mm^3), tend to show higher Treg percentages among $CD4^+$ T cells (although with high inter-individual variability) comparing to healthy controls and that those patients under HAART tend to normalize Treg percentage. However, after 2 years (longitudinal study) or even more years (cross-sectional study) of HAART, although the median Treg percentage of HIV-infected patients was not different when compared with that of healthy individuals, the number of HIV-infected patients with a Treg percentage ≥10% was significantly superior to the one within the healthy group, and patients still demonstrated a more heterogeneous distribution of Treg

percentages. We also observed that a Treg subset homeostasis disturbance (lower naïve Treg and higher cycling Treg among Treg) was present at baseline and that this disturbance had not normalized even after two years under HAART in patients in a more advanced stage of the disease (<200 $CD4^+$ T cell/mm^3).

Concerning immune recovery under HAART, we found, in our cross-sectional study: that amongst the patients with nadir values <200 cells/mm^3, the individuals with higher Treg percentages had the poorest $CD4^+$ T cell reconstitution; that the well-described direct correlation between the nadir value and $CD4^+$ T cell reconstitution was clearly more evident in individuals with high Treg proportions; and finally, we also found a strong negative correlation between Treg percentages and $CD4^+$ T cell recovery among immunological non-responder HIV-infected individuals. In addition, in our longitudinal study, we confirmed a negative correlation between baseline Treg proportion and $CD4^+$ T cell counts at 24 months of therapy. However, we found that the individuals who presented high Treg percentages at baseline are for the most part the ones with lower nadirs and that there was a link between higher Treg percentage and lower $CD4^+$ T cell counts at baseline that explained the correlation found ($CD4^+$ T cell counts progression during therapy was independent on having high or low Treg percentages at baseline).

One of the patients of the longitudinal cohort has developed a paradoxical toxoplasmosis-IRIS therefore allowing us to investigate the Treg and Treg subsets dynamics before, throughout, and after the process. We found an accentuated deregulation of Treg percentage and Treg subsets which suggests that these cells might have an important role in that condition.

Células T CD4+ Reguladoras na Reconstituição Imune de Indivíduos Infetados por VIH

Resumo

A Síndrome de Imunodeficiência Adquirida (SIDA) causada pelo vírus da imunodeficiência humana (VIH) é caracterizada por um desequilíbrio marcado do sistema imunitário, principalmente ao nível da imunidade mediada por células, levando a uma fase final de incapacidade para contrariar um grande número de infeções e tumores. Com o advento da terapêutica antirretrovírica de alta eficácia (HAART), a infeção pelo VIH tornou-se numa doença crónica controlável. No entanto, a HAART não restaura completamente a saúde e nem sempre é suficiente para que os doentes recuperem totalmente as células T CD4+ para valores que possam ser considerados normais.

As células T reguladoras (Treg), uma subpopulação de células T especializadas em regular a resposta imunitária de outras células, desempenham um papel essencial no controlo da resposta imunitária. No contexto da infeção pelo VIH, as Treg podem ser consideradas prejudiciais por poderem diminuir ou mesmo suprimir as respostas imunitárias específicas anti-VIH ou benéficas, ao conseguirem diminuir a ativação imunitária exagerada encontrada no contexto desta doença. Tem sido também defendido que a sua desregulação, frequentemente observada em doentes com infeção por VIH, poderia dificultar a recuperação imunitária ou ainda estar envolvida na patogénese da síndrome inflamatória de reconstituição imunitária (IRIS). Apesar dos muitos estudos realizados nesta área, o papel das Treg na infeção pelo VIH ainda é controverso. Esclarecer o seu papel nesta doença é de suma importância para se perceber melhor a reconstituição imunitária e para, potencialmente, se poder usar essa informação na melhoria do manuseamento da doença.

Com o objetivo de compreender o papel das Treg na recuperação imunitária em doentes sob HAART, realizámos um trabalho que envolveu doentes infetados pelo VIH sob HAART.

Foi realizado um estudo transversal que envolveu doentes infetados pelo VIH, todos sob HAART, e todos com bons resultados em termos de diminuição da carga viral, e um estudo longitudinal que envolveu doentes infetados pelo VIH, naïves em terapêutica e que foram seguidos desde o início do tratamento, durante vários momentos até atingirem os dois anos de terapêutica (desde que aderentes à HAART e sendo esta, eficiente na redução da carga viral).

Estes estudos mostraram-nos que doentes infetados pelo VIH não tratados e que se encontram numa fase mais avançada da doença (<200 células T CD4+/mm^3), apresentam percentagens

elevadas de Treg (embora com grande variabilidade inter-individual), em comparação com controlos saudáveis. Observámos também que a HAART tende a normalizar essas percentagens de Treg, ao longo do tempo nesses doentes. No entanto, mesmo após dois (no estudo longitudinal) ou mais anos (no estudo transversal) de HAART, apesar da mediana da percentagem de Treg dos doentes infetados pelo VIH não ser diferente daquela apresentada pelos controlos saudáveis, o número de doentes infetados pelo VIH que apresentavam uma percentagem de Treg ≥10% era significativamente maior que no grupo controlo e os doentes continuavam a apresentar uma maior variabilidade das percentagens de Treg. Observámos também que a desregulação da homeostasia das Treg (uma menor percentagem das Treg naïves e uma maior proliferação das Treg dentro das Treg) já presente nos doentes antes de iniciarem HAART, não normaliza com a terapêutica, nos doentes em fase mais avançada de doença (<200 células T $CD4^+/mm^3$), persistindo alterada mesmo ao fim de dois anos de terapêutica antirretrovírica.

No que diz respeito à recuperação imunitária sob HAART, encontrámos, no nosso estudo transversal: que entre os doentes com valores de nadir <200 células/mm^3, os indivíduos com percentagens altas de Treg tiveram pior reconstituição de células T $CD4^+$; que a correlação direta já bem descrita entre o valor do nadir e o de células T $CD4^+$ era claramente mais evidente em indivíduos com percentagens elevadas de Treg; e, finalmente, encontrámos também uma forte correlação negativa entre as percentagens de Treg e a recuperação de células T $CD4^+$ entre os indivíduos infetados pelo VIH, imunologicamente não respondedores. Além disso, no nosso estudo longitudinal, encontrámos uma correlação negativa entre a percentagem de Treg no momento do início da terapia e o valor das células T $CD4^+$ aos 24 meses de terapêutica. No entanto, verificou-se que os indivíduos que apresentavam percentagens altas de Treg no início do estudo eram, na maior parte, aqueles com valores de nadir inferiores e que era o elo entre a alta percentagem de Treg e o baixo valor de células T $CD4^+$ no início do estudo, que explicava a correlação encontrada (a progressão da contagem de células T $CD4^+$ durante a terapia era independente de se ter percentagens de Treg altas ou baixas no início do estudo).

Um dos doentes da coorte longitudinal desenvolveu uma IRIS paradoxal no contexto de uma toxoplasmose, permitindo-nos observar a dinâmica das Treg e das suas subpopulações celulares antes, durante e depois dessa intercorrência. Constatámos uma desregulação acentuada da percentagem das Treg e das suas subpopulações durante essa intercorrência, o que nos sugere que as Treg possam ter um papel importante nesse processo.

Table of Contents

Abbreviation List

ABC	Abacavir
ADCC	Antibody-dependent cellular cytotoxicity
AIDS	Acquired Immunodeficiency Syndrome
aPBS	Apyrogenic phosphate buffered saline
APC	Antigen presenting cell
ART	Antiretroviral therapy
ATV	Atazanavir
AZT	Zidovudine
BTLA	B and T lymphocyte attenuator
cAMP	Cyclic adenosine monophosphate
CCR5	CC chemokine receptor Type 5
CD	Cluster of differentiation
CDC	Centers for disease control and prevention
CHP	Centro Hospitalar do Porto
CMV	Cytomegalovirus
CNS	Central nervous system
COBI	Cobicistat
CRFs	Circulating recombinant forms
CSF	Cerebrospinal fluid
CT	Computer tomography
CTL	Cytotoxic $CD8^+$ T lymphocytes
CTLA-	Cytotoxic T-lymphocyte associated antigen 4
CXCR4	CXC chemokine receptor type 4
DC	Dendritic cell
ddI	Didanosine
DNA	Deoxyribonucleic acid
DRV	Darunavir
DTG	Dolutegravir
EACS	European AIDS clinical society
EC	Elite controllers

ECS	School of health sciences
EDTA	Diaminoethanetetraacetic acid
EFV	Efavirenz
EVG	Elvitegravir
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FOXP3	Forkhead box P3
FPV	Fosamprenavir
FTA-ABS	Fluorescent treponemal antibody absorption
FTC	Emtricitabine
GALT	Gut-associated lymphoid tissues
GARP	Glycoprotein A repititions predominant
GITR	Glucocorticoid-induced TNFR-related protein
HAART	Highly active antiretroviral therapy
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDACs	Histone deacetylases
HIV	Human immunodeficiency virus
HIVAN	HIV associated nephropathy
HJUU	Hospital Joaquim Urbano Unity
HLA-B	Major histocompatibility complex, class I, B
HLA-DR	Human leukocyte antigen
HSV	Herpes simplex virus
HVEM	Herpesvirus entry mediator
IAS	International AIDS Society
IFN	Interferon
ICOS	Inducible costimulador
ICVS	Health Sciences Research Institute
IDO	Indoleamine 2,3-dioxygenase enzyme
IGRA	IFN-gamma release assay
IL	Interleukin
INSTI	Integrase strand transfer inhibitor

IPEX	Immune deregulation polyendocrinopathy enteropathy X-linked
IQR	Interquartile range
IRIS	Immune reconstitution inflammatory syndrome
IVDUs	Intravenous drug users
Lag-3	Lymphocyte activation gene 3
LFA-1	Lymphocyte function-associated antigen 1
LIP	Lymphopenia-induced proliferation
LMP	Progressive multifocal leukoencephalopathy
LTNP	Long-term non-progressors
LPV	Lopinavir
LTR	Long terminal repeat
MAC	*Mycobacterium avium complex*
MALT	Mucosa-associated lymphoid tissue
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
MSM	Men having sex with others men
MVC	Maraviroc
NK	Natural killer cell
NKT	Natural killer T cell
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside analogue reverse transcriptase inhibitor
NVP	Nevirapine
PAMP	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCNSL	Primary central nervous system lymphoma
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PD-L	Programmed death ligand
PET	Positron emission tomography scan
PGL	Persistent generalized lymphadenopathy
PI	Protease inhibitor

PPR	Pathogen recognition receptor
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RA	Retinoic acid
RAL	Raltegravir
RNA	Ribonucleic acid
RPV	Rilpivirine
RTE	Recent thymic emigrant
/r	Ritonavir boosted
SD	Standard deviation
SIV	Simian immunodeficiency virus
STAT5	Signal transducer and activator of transcription 5
SQV	Saquinavir
TCR	T cell receptor
TDF	Tenofovir
TGF	Transforming growth factor
TH	T helper cells
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	Tumor necrosis factor receptor
Treg	Regulatory T cells
TSP	Thrombospondin
TST	Tuberculin skin test
UM	University of Minho
URFs	Unique recombinants forms
USDHHS	United States Department of Health and Human Services
VDRL	Venereal disease research laboratory test,
WHO	World Health Organization
ZDV	Zidovudine
3TC	Lamivudina

General Objectives and Outline of the Thesis

Acquired Immunodeficiency Syndrome (AIDS), a cellular immunodeficiency caused by the human immunodeficiency virus (HIV), was first recognized in 1981 and dramatically evolved to a global pandemic, with cases reported from virtually every country. Globally, an estimated 35.3 (32.2–38.8) million people were living with HIV in 2012. There were 2.3 (1.9–2.7) million new HIV infections worldwide, and the number of deaths due to AIDS were estimated at 1.6 (1.4–1.9) million in the same year.

Great advances were made until the present in the HIV field. The advances made are a result of science, advocacy, political commitment, and effective partnerships with affected communities. HIV infection, considered in the beginning a rapidly fatal disease, is now considered a chronic disease easy to stabilize, at least, when the patients are early diagnosed, where the therapy and laboratory support are available, and when patients adhere to treatment and health care.

Some issues related to this infection remain however a problem, even if patients are maintained many years under effective therapy; reservoirs where persistence of virus is the most important impediment to achieve a complete eradication of the virus; the persistence of a residual immune activation and inflammation accounting for a number of non-AIDS-related co-morbidities, and also the inability of some patients to completely immune reconstitute being more prone to suffer from AIDS-related and non-AIDS-related complications and death.

In an attempt to better understand the role of various interveners, particularly the human regulatory $CD4^+$ T cells (Treg), during the immune reconstitution, we endeavor a work with patients selected from Hospital Joaquim Urbano Unity of Centro Hospitalar do Porto, Porto, Portugal (HJUU/CHP) where these patients underwent medical care and from whom additional blood samples were analyzed at Life and Health Sciences Research Institute, School of Health Sciences, University of Minho (ICVS/ECS/UM). Part of the work developed culminated in this thesis.

The present dissertation is organized in three chapters: Introduction; Results; and Final remarks, general discussion and conclusion.

In **Chapter 1**, a general introduction is presented describing the state of art concerning both infection by HIV, and Treg. In the subchapter concerning infection by HIV, an overview is made on several aspects such as: the virus and its replication cycle; the natural history of the

infection covering transmission, pathogenesis and clinical spectrum; and the alteration of the natural history of the infection by highly active antiretroviral therapy (HAART) administration with the consequent immune reconstitution. Some considerations related to incomplete immune-response that can arise despite efficacious antiretroviral therapy are also made in this subchapter. In the subchapter related to Treg, a revision of the theme is made trying to address several issues about these cells as where do they come from, how are they characterized and how do they work, and what is their role in infections and in particularly, HIV infection.

In **Chapter 2**, results are presented in four subchapters:

In **the First subchapter**, we explain how we gather a set of clinical, imaging, laboratory, and biologic data from HIV-1 infected patients; how patients were selected, what clinical and laboratorial data were collected from the hospital, and what supplementary analyses were made at ICVS/ECS/UM. Also a characterization of the study populations at baseline is showed in this subchapter. In this subchapter, the potentiality of such a set of data is also presented.

The Second subchapter is composed of a paper, published in Plos One, entitled "Poor Immune Reconstitution in HIV-Infected Patients Associates with High Percentage of Regulatory $CD4^+$ T Cells". In a cross-sectional study, high Treg percentages were shown to be associated with sub-optimal $CD4^+$ T cell recovery. This was particularly relevant for immunological non-responders with low nadir values. This work suggested that the Treg proportion may be of clinical relevance to somehow predict the immunological reconstitution, at least in patients presenting low $CD4^+$ T cells nadirs at the HAART initiation.

The Third subchapter is presented in the form of article prepared for submission. In a longitudinal study performed to better knowledge the temporal order of events taking place in the immune reconstitution, we found that individuals, naïve for HAART, with lower $CD4^+$ T cell counts, not only showed a higher Treg frequency median, but also a higher range of Treg frequencies, and both the Treg proportion and the range of those values decrease and tend to adjust to those of controls over time under HAART. Moreover, we found that individuals with higher Treg percentages at baseline have lower $CD4^+$ T cell counts after 24 months of HAART. But, we also found that a higher Treg frequency at baseline was only an indirect predictor of a lower $CD4^+$ T cell count at 24^{th} month of antiretroviral therapy as that high Treg percentage was linked to a lower $CD4^+$ T cell count at baseline. As the absolute increase in the $CD4^+$ T cell counts and the reconstitution $CD4^+$ T cell counts slope are constant and independent of $CD4^+$ T cell counts at baseline, those patients with lower $CD4^+$ T cell counts at baseline, show lower $CD4^+$ T cell counts

constantly over time under therapy. Thus, the apparently negative impact of regulatory T cells in $CD4^+$ T cells recovery (showed in the second subchapter) was not verified in a longitudinal study, being that apparent association explained by the existing linkage between low $CD4^+$ T cell counts and high Treg percentages. Interesting alterations on the sub-populations of the Treg are shown and it is highlighted how low nadirs disturb the immune system at distinct levels.

In **the Fourth subchapter**, we present a clinical case report of a patient included in our database, which is strongly suggestive of a paradoxical toxoplasmosis-immune reconstitution inflammatory syndrome with the involvement of the spinal cord. The rarity, not only of the immune reconstitution inflammatory syndrome related with toxoplasmosis, but also the localization of the lesions (spinal cord), renders this presentation a case of particular interest. We investigated a linkage between this immune reconstitution inflammatory syndrome and the $CD4^+$ T Treg frequency on $CD4^+$ T cells, and Treg subsets dynamics over time before and after antiretroviral therapy onset.

Finally in the last and **Third Chapter**, the general discussion of the work developed, as well as the final remarks and conclusion are presented.

Chapter 1

General Introduction

1.1 Infection by the Human Immunodeficiency Virus (HIV) and the Acquired Immunodeficiency Syndrome (AIDS)

1.1.1. Introduction

Acquired Immunodeficiency Syndrome (AIDS) was first recognized in homosexual men in the summer of 1981, in the United States [1,2]. Shortly after, the disease was identified in male and female injection drug users; in hemophiliacs and blood transfusion recipients; among female sexual partners of men with AIDS; and among infants born to mothers with AIDS [3]. By 1983, it was demonstrated clearly that a virus, later named – the human immunodeficiency virus (HIV) – was the causative agent of this new acquired cellular immunodeficiency [4] that dramatically evolved to a global pandemic, with cases reported from virtually every country [5]. More than three decades after its recognition, an enormous amount of information in the areas of HIV virology, pathogenesis, treatment, and prevention has been flowed and continues expanding.

Although two types of HIV are currently recognized, HIV-1 and HIV-2, HIV-1 is responsible for the vast majority of cases. HIV-2 maintains a much more restricted geographical distribution, predominantly in West Africa nations, namely Republic of Guinea-Bissau, Gambia, Senegal, Cape Verde, Ivory Coast, Mali, Sierra Leone, and Nigeria. HIV-2 infection has also been reported in European countries, especially the ones with strong historical and socio-economic ties to West Africa as France and Portugal [6]. Compared with HIV-1 infection, asymptomatic infection is more common in HIV-2-infected individuals, blood virus loads tend to be lower, and transmission to partners or neonates is less frequent [7].

Globally, an estimated 35.3 (32.2–38.8) million of people were living with HIV in 2012. There were 2.3 (1.9–2.7) million new HIV infections globally, and the number of AIDS deaths was 1.6 (1.4–1.9) million in the same year. Since its discovery, AIDS has caused an estimated 36 million deaths worldwide (prevision up to 2012) [5].

In Portugal, at the end of 2012, 42580 cases of HIV infection were reported cumulatively (1436 - 3,4% - corresponded to HIV-2 infection). In 2012, there were 776 new cases diagnosed (32 - 4,1% - of which were caused by HIV-2) and 139 AIDS-related deaths [6]. Porto was the second district of Portugal, the first being Lisbon, presented with the largest cumulative number of cases - 8637 (20.3%). Hereafter, HIV-1 will be only referred and designated as just HIV.

1.1.2. Infection by HIV

1.1.2.1. The Main Characteristics of the HIV

The HIV belongs to *Lentivirus* genus, *Orthovirinae* subfamily and *Retroviridae* family of virus [7,8,9]. The HIV is an enveloped roughly spherical virus that is 80 to 120 nm in diameter (Figure 1) and encloses a capsid containing two identical copies of the virus genome composed by positive-strand RNA. The virion contains several copies of viral enzymes as the reverse transcriptase, protease and integrase [7,8,9]. The genome of this virus consists of three major structural genes, and other additional genes (Figure 2). The three major structural genes, namely *gag, pol* and e*nv*, encode for structural and enzymatic proteins: *gag* encodes for the proteins that form the core of the virion *(capsid* – p24*, nucleocapside* – p7*, matrix* – p17*,* and *nucleic acid-binding proteins); pol* encodes for the enzymes responsible for protease processing of viral proteins, reverse transcription, and integration *(protease, reverse transcriptase, and integrase);* and *env* encodes for the envelope glycoproteins (three pairs of gp120/gp41 forming a trimeric structure). Variations in the gene *gag* or *env* originate the different classes, subtypes or subsubtypes. The other genes, some of whose proteins are essential to regulate viral replication, are: *tat, rev, nef, vif, vpr,* and *vpu*. At each end of each of the RNA strands are long terminal repeat (LTR) sequences that contain promoters, enhancers, and other genetic sequences used for binding different cellular transcription factors [7,8,9].

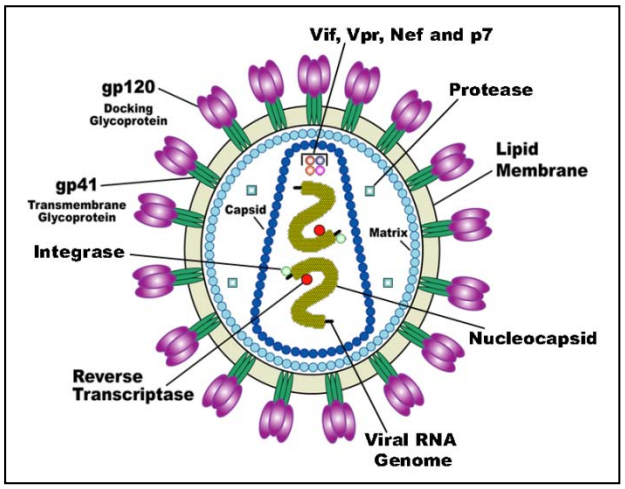

Figure 1. Organization of the HIV virion (extracted from [10]).

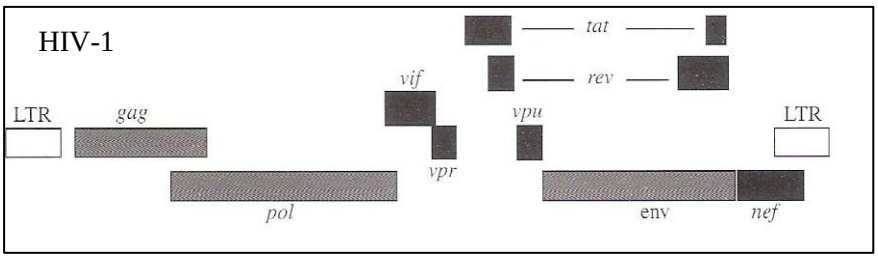

Figure 2. Genomic organization of HIV: the typical three structural genes (*gag, pol* and *env*), the six accessory genes (*tat, rev, vif, nef, vpr,* and *vpu*) located mainly between the *pol* and *env* genes, and the long terminal repeat (LTR) located at each end of the RNA strand (extracted from [11]).

There are four major classes of HIV [12]: M, N, O, and P. Among the virus from the M class, which account for more than 90% of HIV infection worldwide, nine subtypes (or clades) designated by the letters A-D, F-H, J, and K followed by the number correspondent to the six subsubtypes A1-A4 and F1-F2 became recognized. The infection of a cell by two or more viruses may generate recombinant forms designated by CRF *(circulating recombinant forms* – forms that give rise to new epidemic outbreaks) or URF *(unique recombinant forms)*, both nominated by a number (indicating the discovery order) and letters (indicating the subtypes involved), for example CRF01_AE corresponding to the first CRF described that result from a recombination between A and E subtypes. When more than two subtypes are involved, the CRFs are designated as CRF_cpx (complex) [reviewed in 11]. The clade B, that accounts for only 12% of the infection worldwide, is de most common subtype in the Americas and Western Europe. The subtypes non-B (A, C, D, F-H, J, and K) account for the great majority of the infections in the rest of the world being the subtype C the most prevalent (responsible for more than 50% of infections worldwide) [11]. As in the American continent, the majority of European isolates of HIV belongs to subtype B. However, several countries including Portugal, also reported appreciable numbers of infections by non-B subtypes, with particular relevance to the subtype G, C and CRF03-AB [11 and references therein].

1.1.2.2. The Replication Cycle of HIV

The replication cycle of HIV (Figure 3) [7,8,9,11] begins with the high-affinity binding of the gp120 protein (a viral envelope external protein) to its privileged receptor on the host cell surface, the CD4 molecule. The CD4 is a protein expressed predominantly by a subset of T lymphocytes, the $CD4^+$ T cells or helper T cells, but also expressed on the surface of monocytes/macrophages, microglia, follicular dendritic cells of lymphoid tissue, blood dendritic cells or their homologues as the Langerhans cells (in skin and mucosa). The binding of the virus

to the CD4 on the cell surface triggers a conformational change in gp120 allowing the virus to bind to a co-receptor (CC chemokine receptor type 5 - CCR5 - or CXC chemokine receptor type 4 - CXCR4) also expressed on the host cell surface. Following this initial step, the fusion domain of HIV gp41 envelope protein is exposed and the fusion of the virus envelope and cellular membrane occurs followed by the virus entrance into the target cell. The virus genome, released from the capsid, serves as a template to the synthesis of a complementary (the early phase of replication begins) negative-strand DNA with the participation of the viral reverse transcriptase (RNA-dependent DNA polymerase) and a transfer RNA (tRNA) as a primer. The reverse transcriptase is also responsible for the destruction of the template RNA and by the construction of the second complementary strand of DNA. A double-stranded DNA is then formed and transported into the nucleus (as a complementary DNA - cDNA). The cDNA or "the proviral DNA" is integrated into the host chromatin with the aid of the viral integrase enzyme that is also transported to the nucleus, thereafter behaving almost like a cellular gene.

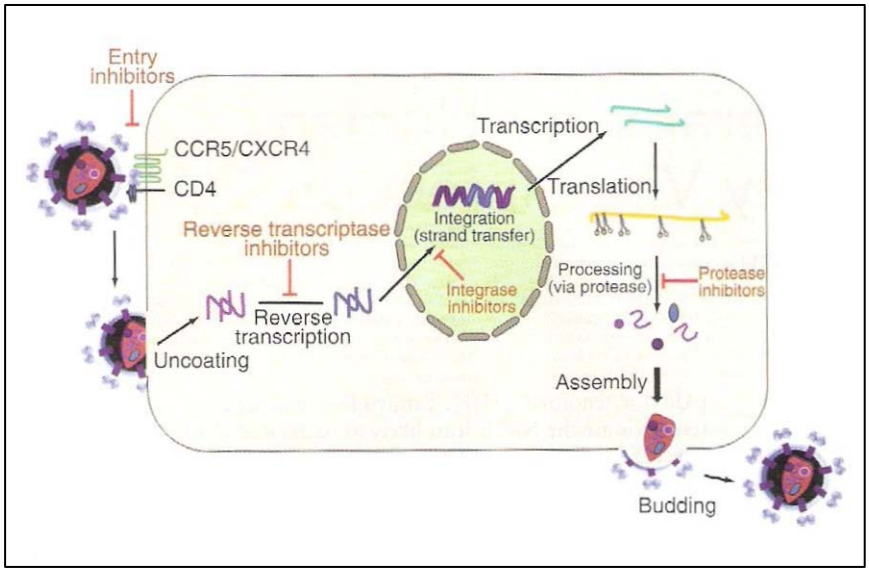

Figure 3. Replication cycle of HIV with different sites of action of antiretroviral agents (extracted from [7]).

Cellular activation plays an important role and is required in the efficient integration and to the initiation of the late phase of replication. This late phase begins when the proviral DNA is transcribed by the host RNA polymerase II, and is regulated by the interaction of host transcription factors with promoter and enhancer elements in the LTR portion of the viral genome. Both genomic RNA and several mRNA are then produced. The six accessory viral gene products, in concert with the cellular machinery, regulate the HIV replication in different stages. Newly generated HIV core proteins, enzymes, and genomic RNA assembly inside the cell, and

immature viral particles form and bud off from the cell, acquiring their envelope from the cell membrane. Yet, the core of the virus is still immature. Final morphogenesis of HIV requires protease cleavage of *gag* and *gag-pol* polypeptides that continues even after envelopment. Infectious viral particles are then ready to infect other cells [7,8,9,11].

1.1.3. Natural History of the HIV Infection

1.1.3.1. HIV Transmission

The HIV pandemic is still in a dynamic phase in most parts of the world, with continuing geographic spread and changing epidemiologic patterns. As already noted, AIDS was initially described in young, promiscuous, homosexual men [1,2] and is still prevalent in the gay community. However, heterosexual transmission by vaginal intercourse and parenteral transmission by intravenous drug abuse (from sharing contaminated syringe needles) have become the major routes by which HIV is being spread. Therefore, it can be said that HIV is transmitted primarily by sexual contact (both anal and vaginal intercourse); by blood and blood products; and by infected mothers to infants intrapartum, perinatally, or via breast milk [7,8,9,11].

In Portugal, by December 2012, the transmission category that cumulatively recorded the highest number of cases was the "heterosexual category", followed by "intravenous drug user category" and “homo/bisexual category” corresponding to, respectively, 44.6%, 38.7% and 14.1% of total cases notified [6]. Sexual transmission was thus observed in the majority (58.7%) of reported cases [6]. The heterosexual transmission has assumed greater importance in recent years and in very recent years, an increase in the number of cases referring to homosexuals has also been noticed [6]. It is important to take into account that activities undertaken by the responsible entities for drug prevention had made impact over time, reducing the number of cases associated with intravenous drug use [6].

1.1.3.2. The Acute Retroviral Syndrome

Following an exposure of a mucosal surface to HIV, the virus can cross the barrier by binding to dendritic or Langerhans cells, or through microscopic rents in the mucosa [8]. Viral replication has been shown to occur locally for virus infecting $CD4^+$ T cells that although spatially dispersed and partially activated, may be present at lamina propria [8]. Regardless of the route of

HIV transmission (whether it occurs through a mucosa barrier, or by direct introduction of the virus into the bloodstream), the virus reaches, after about two days of infection, the regional lymphoid organs. It appears that dendritic or other cells of the monocyte-macrophage lineage play an important role in this process of HIV transportation through the blood [13]. In the lymphoid organs, HIV antigens are presented to $CD4^+$ T cells found in a dense concentration, triggering their activation and infection [7,8].

After an active replication in the regional lymph nodes, the spread of the virus throughout the body (to other lymph nodes, gastrointestinal tract, brain, liver, spleen) occurs, leading to a very high viremia and a significant decrease in the blood $CD4^+$ T cell counts [7,8]. An important lymphoid organ, the gut-associated lymphoid tissue (GALT), is a major target of HIV infection due to its location and the local presence of a large number of $CD4^+$ T cells [8], and may be even the first lymphoid tissue to be reached by the virus, and to amplify the virus [13]. The acute retroviral syndrome (the early acute phase of the disease), which coincides with this high viremia, occurs two to three weeks after infection (in Figure 4 is shown the natural history of HIV infection) [14]. At this stage, various unspecific symptoms and signs, acute mononucleosis-like, may occur rendering the recognition of the disease possible in its earlier stage, although, most frequently not recognized. Common symptoms are fever, prostration, lymphadenopathy, sore throat and pharyngitis, erythematous maculopapular rash, arthralgia and myalgia, diarrhea, headache, meningeal syndrome and other neurological signs. Patients recover from this phase spontaneously and seroconvert (anti-HIV antibodies become detectable in the blood) [11]. Antibodies anti-HIV proteins become generally detectable three to six weeks after the development of plasma viremia [8].

1.1.3.3. The Chronic Asymptomatic or "Latency" Phase

The development of a humoral and cellular immune response against HIV leads to the recovery of the first phase and to the progression to the second phase which is known as the chronic asymptomatic or "latency" phase [15,16]. This acquired immune response, although not fully effective, appears to have an important impact on the reduction of the plasma viremia to a “setpoint” level that is highly predictive of the later course of disease progression [17]. The humoral response consists on the production of antibodies against multiple antigens of HIV although the precise functional significance of these different antibodies is unclear. The anti-p24 antibodies appear to contribute to the decline of viremia after acute infection, and the envelope

proteins, gp120 and gp41, are the only viral protein to elicit neutralizing antibodies. These neutralizing antibodies may be protective, neutralizing HIV directly, preventing the spread of infection to additional cells, or participating in antibody-dependent cellular cytotoxicity (ADCC). The cellular response is for the most part mediated by cytotoxic $CD8^+$ T lymphocytes (CTLs). These CTLs, through their HIV-specific antigen receptors, bind to and cause the lytic destruction of HIV-infected cells bearing autologous major histocompatibility complex (MHC) class I molecules presenting HIV antigens. Other $CD8^+$ T cells present the ability of inhibiting viral replication in a non-cytolytic manner, mediated by soluble factors called CD8 antiviric factors [11]. HIV-specific $CD4^+$ T cells are very important in the orchestration of the immune response to HIV by providing help to HIV-specific B cells and $CD8^+$ T cells [8,11]. In addition to T-cell mediated immunity and ADCC, that involves the killing of HIV-expressing cells by Natural Killer cells (NK) armed with specific antibodies directed against HIV antigens, also, NK cells alone have been shown to kill HIV-infected target cells in tissue cultures [8,11].

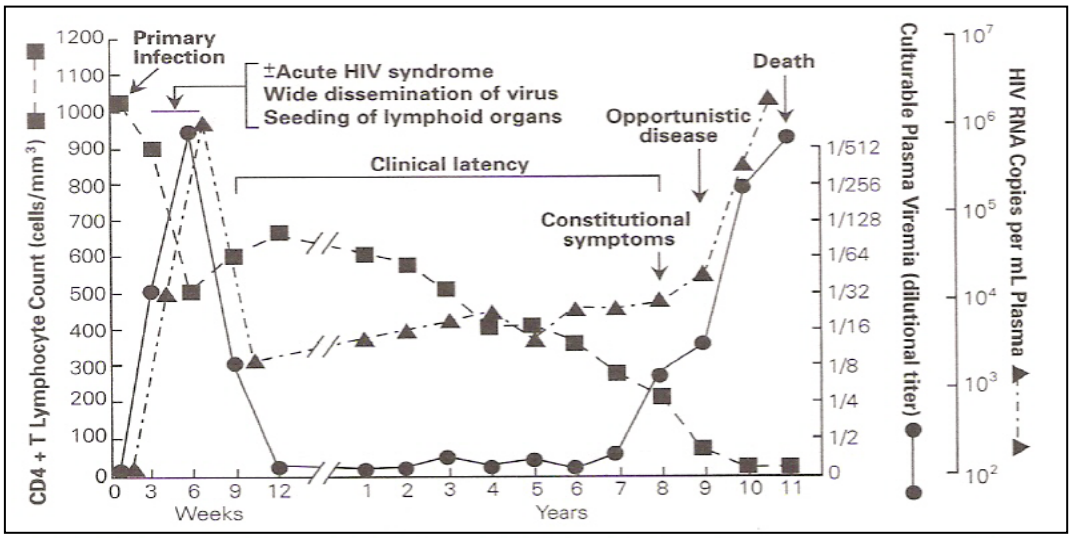

Figure 4. Natural history of HIV infection (extracted from [14]).

The immune response is responsible for infection control for several years (on average 7 to 10 years) [8,11,14]. During this stage, the HIV viral load in the blood remains low, but even during this phase, there is a solid and intense virus replication in the lymph nodes [18]. As the immune system, at this phase, is able to replenish the number of $CD4^+$ T cells that are being destroyed, the number, after the initial recovery, appears to be stable, or slowly decreasing. Thus, this phase, although clinically latent, is not virologically latent. The patients may still show

nonspecific symptoms such as persistent fever, malaise, weight loss or lymphadenopathy [reviewed in 11].

HIV, however, is able to elude the immune response through various mechanisms [reviewed in 8,11,13], namely: 1) the sustained level of replication leads to viral diversity, via mutation or recombination, and consequently, to epitope mutation and evasion of control from CTLs and from neutralizing antibodies; 2) apart from epitope mutation, also the glycosylation of the envelope or conformational masking of neutralizing epitopes contribute to evasion from specific neutralizing antibodies; 3) the production of proteins from *tat* and *nef* genes can change class I MHC molecules that lose their ability to present HIV epitopes rendering the cytotoxic $CD8^+$ T cells less able to recognize HIV-infected cells; 4) the down regulation of MHC-II molecules that renders $CD4^+$ T cells less able to effectively participate in the immune response; 5) the exhaustion, dysfunction and death of immune cells that render the immune response weaker; and finally, 6) the presence of infected cells that are not recognize as infected by the immune system, due to their state of latency (not expressing antigens) or to their sequestration in immunologically privileged sites such as the central nervous system, contribute to viral persistence. All these mechanisms lead to the gradual increase of the HIV viral load and the steady reduction in the number of $CD4^+$ T cells with the consequent reduction of cellular immunity and the gradual evolution to the next and final stage of the disease.

1.1.3.4. Mechanisms Responsible for the Reduction of $CD4^+$ T Cells Numbers in HIV-Infected Patients

There is considerable controversy regarding the relative contribution of the diverse mechanisms responsible for the reduction of the $CD4^+$ T cells during the course of HIV infection that occur through increased destruction, decreased production, and redistribution [reviewed in 8,13,19,20].

Reduction of the $CD4^+$ T cells numbers results in part from the direct effects of infection of $CD4^+$ T cells through several mechanisms [reviewed in 19] like: the disruption of the plasma membrane owing to the continuous budding of the virions or due to the enhanced permeability induced by viral *vpu;* increased cellular toxicity by the accumulation of nonintegrated viral DNA; the alteration by HIV of the normal cellular balance of pro- versus anti-apoptotic proteins resulting in the disruption of mitochondrial function and death of the cell; and the short lifespan of giant multinucleated cell called syncytium (formed by membrane fusion between infected cells that

express gp120 and the CD4 molecule expressed on the surface of all $CD4^+$ T cells). Apart from the cytopathic effects of HIV, $CD4^+$ T cells infected by HIV are also directly killed by HIV-specific cytotoxic $CD8^+$ T cells, by ADCC, or directly by NK as referred above [8,13,19,20].

The infection of $CD4^+$ T cells, however, does not appear to be sufficient to explain the progressive loss of $CD4^+$ T cells which occurs over the course of HIV disease. In fact, the number of apoptotic cells in infected individuals greatly exceeds the number of HIV-infected cells indicating that uninfected cells are also killed by bystander indirect detrimental effects of HIV infection [8,13,19,20]. Uninfected cells may be eliminated primarily by [reviewed in 8,13,19,20] Fas-mediated or TNF-inducing ligand mediated apoptosis; due to the down-regulation of anti-apoptotic proteins; or as a result of apoptosis stimulated by HIV proteins (such as gp120, *tat, nef* and *vpu*) released from infected cells [19]. An increased proliferation/turnover as well as death of $CD4^+$ T cells is observed [21]. The excessive immune activation consistently observed seems to be a major determinant of this scenario [13,19,20,22-25]. Indeed, it is now accepted worldwide that the constant stimulation of the immune system plays a pivotal role in the progression from HIV infection to AIDS [22]. This activation of the innate and adaptive immune system leads to the release of pro-inflammatory mediators (namely TNF, IL-1β, and IL-6) which drives persistent inflammation [26]. This induced activation inflammatory condition, in addition of driving $CD4^+$ T cell depletion, as already mentioned, may be responsible for the premature age-associated changes in the immune system (immunosenescence) demonstrated in HIV-infected patients (as many of the abnormalities in T cells observed in older adults are similar to those observed in untreated HIV-infected individuals), and for a number of co-morbidities non-AIDS related (to be referred later in this chapter) [26,27]. Several mechanisms may drive this immune activation such as [reviewed in 26,28]: ongoing HIV replication, microbial bioproducts translocation, mostly from the gut, like lipopolysacharide (as a great proportion of the $CD4^+$ T cells pool in the GALT is depleted early in the infection course rendering the immune barrier leaky); increased viral load of co-infecting pathogens (CMV, other herpesvirus, and hepatitis virus), disregulation of regulatory T cells and other immunoregulatory cells, or lymphopenia per se [28].

In addition to the increased destruction of $CD4^+$ T cells, a decreased production of these cells due to the disruption of normal hematopoiesis in the bone marrow and impaired thymus function (owing to several mechanisms) seems also to occur in a great proportion of patients during HIV infection [8,29].

The redistribution caused by trafficking of $CD4^+$ T cells from the peripheral blood to lymphoid tissue or inflammatory extra-lymphoid tissues could, in addition, account as an additional mechanism for $CD4^+$ T cell peripheral blood depletion in HIV-infected patients [21].

It is important to refer that the deleterious effects and immune dysfunction due to HIV infection are extended to several other cells of the immune system like $CD8^+$ T, NK, B, nonlymphoid cells like macrophages and dendritic cells [20].

1.1.3.5. The Stage of Immunodeficiency or AIDS

The gradual imbalance that occurs in the immune response, mainly in cell-mediated immunity leads early to the occurrence of infections that do not endanger the patient's life [those former designed by the Center for Disease Control and Prevention (CDC) as type B clinical category] [30], and later, to major life threatening infections/conditions that mark the transition of the disease to the final stage - the stage of immunodeficiency or AIDS. This phase results, if antiretroviral therapy is not administered, in the death of the individual in about one to three years, due to the appearance of several opportunistic infections and tumors. This stage occurs essentially when the $CD4^+$ T cell counts in the blood are below 200 cells/mm^3 (Table 1 shows the correlation of HIV-related complications occurrence with the $CD4^+$ T cell counts) [31].

To improve standardization and comparability of surveillance data regarding people at all stages of HIV disease, the CDC proposed a classification system for adolescents and adults infected by HIV that was updated in 2008 [32]. This ranking is based on the number of $CD4^+$ T cells per mm^3 of blood (or their percentage in the total blood lymphocytes) and the clinical history of the patient as described in Table 2 [32].

As mentioned before, other clinical conditions (non-AIDS-defining complications), in addition to opportunistic infections and tumors (AIDS-defining complications), occur and contribute to morbidity and mortality seen in these patients. These non-AIDS co-morbidities, many of which are similar to those observed among the elderly, include cancer, heart, liver, kidney and bone disease, frailty, and neurocognitive decline, and provide indirect evidence that HIV infection might accelerate the aging process [26].

CD4+ T cell counts (cells/mm³)	Infectious Complications	Noninfectious Complications
> 500	- Acute retroviral syndrome - Candidal vaginitis	- Persistent generalized lymphadenopathy (PGL) - Guillain-Barré syndrome - Myopathy - Aseptic meningitis
200 – 500	- Pneumococcal and other bacterial pneumonia - Pulmonary tuberculosis - Herpes zoster - Oropharingeal candidiasis - Cryptosporidiosis self-limited - Kaposi's sarcoma - Oral hairy leukoplakia	- Cervical and anal dysplasia - Cervical and anal cancer - B-cell lymphoma - Anemia - Mononeuronal multiplex - Idiopathic thrombocytopenic purpura - Hodgkin lymphoma - Lymphocytic interstitial pneumonia
< 200	- *Pneumocystis* pneumonia -Disseminated histoplasmosis and coccidioidomycosis - Miliary/extrapulmonary tuberculosis - Progressive multifocal Leukoencephalopathy (LMP)	- Wasting - Peripheral neuropathy - HIV-associated dementia - Cardiomyopathy - Vacuolar myelopathy - Progressive polyradiculopathy - Non-Hodgkin's lymphoma
< 100	- Disseminated herpes simplex - Toxoplasmosis - Cryptococcosis - Cryptosporidiosis (chronic) - Microsporidiosis - Candidal esophagitis	
< 50	- Disseminated cytomegalovirus (CMV) - Disseminated *Mycobacterium avium complex* (MAC)	- Primary central nervous system lymphoma (PCNSL)

Table 1. Correlation of complications with CD4+ cell counts (adapted from [31]); some conditions listed as "noninfectious" are associated with transmissible microbes as lymphoma – Epstein-Barr virus and cervical and anal cancers – human papillomavirus).

Stage	CD4+ T cell counts cells/mm³ (% of total lymphocytes)	Clinical
1	≥ 500 (≥ 29%)	No AIDS-defining conditions
2	200-499 (14 - 28%)	No AIDS-defining conditions
3	< 200 (< 14%)	Or documentation of AIDS-defining conditions
Unknown	No information	No information

Table 2. Surveillance case definition for HIV infection among adults and adolescents (aged≥ 13 years); (adapted from [32]). Individuals classified in the shaded area meet the criteria for AIDS. AIDS-defining conditions: Candidiasis of bronchi, trachea, or lungs; Candidiasis of esophagus; Cervical cancer, invasive; Coccidioidomycosis, disseminated or extrapulmonary; Cryptococcosis, extrapulmonary; Cryptosporidiosis, chronic intestinal (>1 month's duration); Cytomegalovirus disease (other than liver, spleen, or nodes); Cytomegalovirus retinitis (with loss of vision); encephalopathy HIV related; Herpes simplex: chronic ulcers (>1 month's duration) or bronchitis, pneumonitis, or esophagitis; Histoplasmosis, disseminated or extrapulmonary; Isosporiasis, chronic intestinal (>1 month's duration); Kaposi sarcoma; Lymphoma, Burkitt (or equivalent term); Lymphoma, immunoblastic (or equivalent term);

Lymphoma primary of brain, *Mycobacterium avium complex* or *Mycobacterium kansasii*, disseminated or extrapulmonary; *Mycobacterium tuberculosis* of any site, pulmonary, disseminated, or extrapulmonary; *Mycobacterium,* other species or unidentified species, disseminated or extrapulmonary; *Pneumocystis jirovecii* pneumonia; Pneumonia recurrent; Progressive multifocal leukoencephalopathy; *Salmonella* septicemia, recurrent; Toxoplasmosis of brain; Wasting syndrome attributed to HIV.

1.1.4. Alteration of the Natural History of the HIV Infection

1.1.4.1. Highly Active Antiretroviral Therapy (HAART)

The emergence and administration of drugs with potent antiretroviral activity, and their use in combination (highly active antiretroviral therapy or HAART) to treat patients with HIV infection started in 1996 and markedly reduced the morbidity and mortality associated with this disease.

An effective HAART (the drugs used in such combinations block several steps of the virus replication cycle as shown in Figure 3) is the therapy that conducts to a suppression of the viral load (undetectable viral load in the blood considering the detection limit of commercially available tests). Sustained viral load suppression leads for most patients to the restoration of $CD4^+$ T cell compartment.

In the early times of HAART, regardless of all its related advantages, patients faced complex drug regimens, involving multiple drugs with a variety of administration schedules, high pill burden and troublesome side effects like gastrointestinal symptoms, lipoatrophy, visceral and abdominal fat gain, insulin resistance, DNA damage, or mitochondrial dysfunction. It was, in those times, difficult to maintain the high levels of adherence to therapy, necessary in order to prevent the development of HIV genetic mutations conferring drug-resistant. Since then, huge developments occurred: the design of highly potent drugs; new classes of drugs acting in different steps of the virus replicating cycle; drugs presenting a better safety profile; drugs with longer half-lives allowing once-daily dosing; and the availability of fixed-dose drug combinations [33]. It is now simple to define effective and potent, safe, tolerable, and easy-to-take therapeutic schemes allowing long-term suppression of HIV replication.

The development of laboratory techniques that have been useful to: monitor HIV replication; detect HIV drug resistance; diagnose early and consequently to prevent and/or to treat opportunistic infections more effectively, also contributed to the decline in morbidity and mortality of HIV-infected individuals. HIV infection, considered in the beginning a rapidly fatal disease, is now considered a chronic disease easy to stabilize, at least, when the patients are diagnosed early, where the therapy, laboratory support and health care are available, and most

importantly when patients adhere to treatment. However, one must not forget that, even in face of sustained undetectable viral loads, there are local "sanctuaries"/reservoirs in the body (central nervous system, lymphoid tissue, genital organs) with infected but quiescent non productive of new virus $CD4^+$ T cells. These reservoirs, somehow protected from HAART, are places where transitory replication and viral persistence are possible, allowing the occurrence of viral mutations, contributing to the maintenance of an abnormal level of immune activation, and being the most important impediment to achieve complete eradication of the virus in infected patients, even if they are maintained many years under HAART.

The ideal moment to start HAART for each patient has long been a subject of intense debate. The aim of antiretroviral treatment is not only to prevent $CD4^+$ T cells depletion and preserve its production by suppressing viral replication, but also to reduce the immune activation and persistent inflammation counteracting the immunosenescence and multi-co-morbidities non-AIDS related. Taking this into account, and the existence of safer and comfortable drug schemes, it has been proposed that HAART should be initiated earlier and earlier in the course of the infection [34-36]. However, therapeutic regimen should only be initiated provided that the patient is perfectly elucidated and motivated to adhere to HAART.

In the light of the treatment options currently available, the main scientific societies in the area of HIV [34-36] (European AIDS Clinical Society - EACS, International AIDS Society - IAS, United States Department of Health and Human Services - USDHHS) have their own recommendations for the initiation of antiretroviral therapy to naive patients. Portugal, also edited its own recommendations [37]. The proposed time to start, and initial regimens recommended by each society and by the Portuguese panel are presented in Table 3 [34-37].

When the therapy is initiated at a late stage of the disease, it should be taken into account the possibility that, in the first months and due to immune reconstitution, certain infections, which might have remained silently undiagnosed due to the inactivity of the immune system, or have already been diagnosed and begun to be treated, became symptomatic, or get worse, as the reconstituting immune system might strongly react to these pathogens or antigens [38]. Drug interactions between HAART and other treatments/prophylaxis of opportunistic infections should also be the subject of our attention. The medication intolerance and toxicity seem to occur more often when HAART is initiated in more advanced stages of the disease. Even in this situation, when patients are motivated and adherent to the medication, the immune

reconstitution has been possible, allowing the subsequently discontinuation of primary or secondary opportunistic infections prophylaxis, clear improving their survival and quality of life.

European AIDS Clinical Society [34]	International AIDS Society [35]	United States Department of Health and Human Services [36]
Ideal time to start		
HAART is always recommended in any HIV-positive person with a current CD4+ T cell count below 350 cells/ mm³. For persons with CD4+ T cell counts above this level, the decision to start HAART should be individualized and considered, especially if a person is requesting HAART and is ready to start, has symptomatic HIV disease or various types of (co-morbid) conditions.	HAART is recommended for all adults with HIV infection, regardless of CD4+ T cell counts. The strength of the recommendation* increases as CD4+ T cell count decreases and in the presence of certain conditions, with the following ratings: CD4+ T cell count ≤500 cells/mm³ (AIa); CD4+ T cell count >500 cells/mm³ (BIII). Pregnancy: AIa Chronic hepatitis B virus: AIIa HIV-associated nephropathy: AIIa Acute phase of HIV infection: BIII	HAART is recommended for all HIV-infected individuals to reduce the risk of disease progression. The strength and evidence for this recommendation* vary by pretreatment CD4+ T cell count: CD4+ T cell count <350 cells/mm³ (AI); CD4+ T cell count 350–500 cells/mm³ (AII); CD4+ T cell count >500 cells/mm³ (BIII). HAART also is recommended for HIV-infected individuals for the prevention of transmission of HIV. The strength and evidence for this recommendation vary by transmission risks: perinatal transmission (AI); heterosexual transmission (AI); other transmission risk groups (AIII).
Preferential regimens Rregimens with optimal and durable efficacy, favorable tolerability and toxicity profile, and ease of use.		
A combination of one NNRTI or a ritonavir-boosted PI or an ITI and two NRTI **NRTI:** ABC/3TC (use with caution if HIV RNA>100000 c/ml), FTC/TDF **NNRTI:** EFV, RPV (if HIV RNA<100000 c/ml) **Ritonavir-boosted PI:** ATV/r, DRV/r **INSTI:** RAL, EVG + cobi (+ FTC/TDF) (co-formulated)	**NNRTI-based regimen:** EFV/FTC/TDF; EFV + ABC/3TC; RPV/FTC/TDF (if HIV RNA<100000 c/ml) **PI-based regimens:** ATV/r + FTC/TDF; ATV/r + ABC/3TC; DRV/r + FTC/TDF **INSTI-based regimen:** DTG + TDF/FTC, DTG + ABC/3TC, EVG/cobi/TDF/FTC, RAL + FTC/TDF (the combination ABC/3TC was less efficacious with HIV RNA>100000 c/ml than TDF/FTC when given with EFV or ATZ/r)	**NNRTI-based regimen:** EFV/FTC/TDF **PI-based regimens:** ATV/r + FTC/TDF DRV/r (once daily) + FTC/TDF **INSTI-based regimen:** DTG + ABC/3TC, DTG + TDF/FTC, EVG/cobi/TDF/FTC, RAL + FTC/TDF, **Also recommended if HIV RNA<100000 c/ml:** EFV + ABC/3TC; RPV/FTC/TDF (if CD4>200/mm³) ATV/r + ABC/3TC **Preferred regimen for pregnant women:** ATV/r or LPV/r (twice daily) + ABC/3TC or TDF/FTC or ZDV/3TC
Alternative regimens Regimens that are effective and tolerable but have potential disadvantages compared with preferred regimens. An alternative regimen may be the preferred regimen for some patients.		
Alternative regimen components: **NRTI:** TDF + 3TC, ZDV/3TC, ddl + 3TC or ddl + FTC **NNRTI:** NVP **Ritonavir-boosted PI:** FPV/r, LPV/r, SQV/r **CCR5 inhibitor:** MVC	**NNRTI-based regimen**: NVP + FTC/TDF; NVP + ABC/3TC RPV + ABC/3TC **PI-based regimens:** ATZ/cobi + 2 NRTIs, DRV/cobi + 2 NRTIs DRV/r + ABC/3TC, LPV + 2 NRTIs **INSTI-based regimen:** RAL + ABC/3TC **NRTIs limiting or sparing:** DRV/r + RAL, LPV/r + 3TC, LPV/r + RAL	**PI-based regimens:** DRV/r + ABC/3TC LPV/r + ABC/3TC or FTC/TDF (LPV/r once or twice daily) **INSTI-based regimen:** RAL+ABC/3TC
Beware of remarks and precautions to consider of components and combinations (pregnancy; cardiovascular risk; allele of major histocompatibility complex, class I,B - HLA B*5701; use of proton pump inhibitors). Fixed-dose combinations when possible for patient convenience.		

Portuguese recomendations[37]
Ideal time to start The start of HAART should be individualized and the decision upheld by the following elements: clinical manifestations, number of CD4+ T lymphocytes, plasma viral load value, presence of co-morbidities (AII) and the patient's level of preparedness. The HAART is recommended for all patients with chronic HIV-1 infection with a CD4+ T lymphocytes <350 cells/mm³ (AI). For persons with CD4+ T cell counts above this level, the decision to start HAART should be individualized and considered, especially if a person has symptomatic HIV disease or various types of (co-morbid) conditions, or for the prevention of transmission. (These recommendations are presently under revision – in the next publication HAART will be recommended for CD4+ T cells ≤500/ mm³)
Preferential regimens A combination of one NNRTI or a ritonavir-boosted PI or an ITI and two NRTI **NRTI:** ABC/3TC, FTC/TDF; **NNRTI:** EFV, NVP; **Ritonavir-boosted PI:** ATV/r, DRV/r; **INSTI:** RAL When NVP or RAL are used, they may be combined with FTC/TDF (the next update will recommend also RPV as another NNRTI)
Alternative regimens **NRTI:** ZDV/3TC; **NNRTI:** RPV; **Ritonavir-boosted PI:** FPV/r, LPV/r, SQV/r ; **CCR5 inhibitor:** MVC (the next update will recommend also EVG/cobi as alternative INSTI)

Table 3. Ideal time to start, and initial recommended and alternative regimens in antiretroviral-naïve patients, according to main AIDS societies and Portuguese panel. Adapted from [34-37]. *Definitions for rating/strength of recommendations and the rating/quality evidence: A = Strong; B = Moderate; C = Optional/Limited. Rating of Evidence: Ia – Evidence from≥1 randomized clinical trial (RCT) published in the peer-reviewed literature; Ib - Evidence from ≥1 RCTs presented in abstract form at peer-reviewed scientific meetings; IIa – Evidence from non-RCTs, cohort, or case-control studies published in the peer-reviewed literature; IIb - Evidence from non-RCTs, cohort, or case-control studies presented in abstract form at peer-reviewed scientific meetings; III – Recommendation based on the panel's analysis of the accumulated available evidence (IAS panel); and I = Data from randomized controlled trials; II = Data from well-designed nonrandomized trials or observational cohort studies with long-term clinical outcomes; III = Expert opinion (DHHS panel). NNRTI – non-nucleoside reverse transcriptase inhibitor, NRTI – nucleoside (or nucleotide) analogue reverse transcriptase inhibitors, PI – protease inhibitor, INSTI – integrase strand transfer inhibitor, CCR5 – CC chemokine receptor 5 inhibitor. ABC – abacavir (only for patients who are HLA-B*5701 negative), ATV – atazanavir, cobi – cobicistat, ddI –didanosine, DRV – darunavir, DTG – dolutegravir, EFV – efavirenz, EVG – elvitegravir, FPV – fosamprenavir, FTC – emtricitabine, LPV – lopinavir, MVC – maraviroc, NVP – nevirapine, RAL – raltegravir, RPV – Rilpivirine, SQV – saquinavir, TDF – tenofovir disoproxil fumarate, ZDV – zidovudine, 3TC – lamivudine, /r – ritonavir-booster

1.1.4.2. Immune Reconstitution

Under effective HAART, one assists, not only to the redistribution of memory T cells, but also to the expansion of naïve T cells. The $CD4^+$ T cells restoration takes place during various phases [reviewed in 28,39,40] [Figure 5]. The initial phase of blood $CD4^+$ T cell increment tends to be steeper than in other phases, and occurs in the first six months under HAART. This $CD4^+$ T cells rise is thought to be mostly due to the liberation of memory $CD4^+$ T cells from lymphoid tissues where sequestered (the inhibition of the viral replication leads to a decrease in the immune activation which in turn, drives the down-regulation of adhesion molecules and the liberation of those cells). Two other phases of $CD4^+$ T cell restoration have been defined; the second lasts for most patients up to the end of the second or third year after HAART onset and the third phase may last until seven or more years after HAART initiation, when a $CD4^+$ T cells plateau is reached. [39] [Figure 5]. Although the mechanisms involved in immune reconstitution in the second and third phases are related, the speed of the immune restoration differs between them [Figure 5]; it is quicker in the second (although slower than in the first) than in the third phase. The mechanisms involved in $CD4^+$ T cell restoration in the second and third phases are: increase of thymic output; the decrease of the abnormal rate of cell death allowing the extension of $CD4^+$ T cell half life (the reduction in chronic activation leads to a decreased sensitivity to apoptosis); and proliferation of the residual $CD4^+$ T cells (by homeostatic peripheral proliferation or residual immune activation). Only the first mechanism allows the restitution of the T cell repertoire diversity [40]. As less naïve $CD4^+$ T cells are converting to memory cells, a passive accumulation of those cells occurs in the periphery after HAART initiation also contributing to the immune reconstitution [40].

Several studies have analyzed the immune reconstitution in patient groups with sustained viral suppression under HAART aiming to discover if this was a limited process in time and what are the circumstances that may have hampered T cell reconstitution. The majority of studies show a plateau in $CD4^+$ T cell recovery, in most patients, after 3 to 7 years of complete virological response under HAART, even if the $CD4^+$ T cell counts are still low [40-45]. Even if some patients still have an increase in $CD4^+$ T cells after that period of time, this increase appears to be insufficient for patients that initiate HAART at a very low $CD4^+$ T cell counts to reach normal (700-1100 cells/mm^3) or near normal (>500 cells/mm^3) $CD4^+$ T cell values [41-45]. Others, however, reported continuous reconstitution of these cells after several years of HAART (even upon 10 years of HAART [46]) rendering possible that those normal or near normal values are reached for most patients despite low $CD4^+$ T cells nadirs [46-49]. Nevertheless, in most patients, the $CD4^+$ T cell counts slope as the absolute increase in the $CD4^+$ T cell counts seems to be independent of the severity of the immune alterations present before HAART introduction. Therefore, the time taken to reach normal $CD4^+$ T cells depends to a great extent on the stage of the disease when HAART was initiated [41,47,49,50].

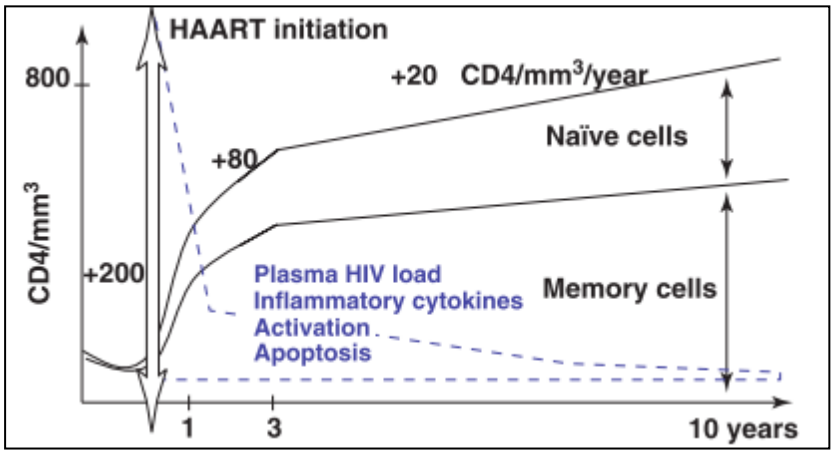

Figure 5. Kinetics and mechanisms of immune reconstitution of $CD4^+$ T lymphocyte compartment after starting HAART (extracted from [40]).

1.1.4.3. Patients with Incomplete Immune Reconstitution

A proportion of HIV-infected patients under HAART, and being virological suppressed during several years (4 - 7 years), do not reach normal (700-1100 cells/mm^3), near normal (> 500 cells/mm^3) or, at least, satisfactory $CD4^+$ T cell counts (higher than 250-350 cells/mm^3, a level that would enable the patient to be protected from the more severe opportunistic infections), and therefore are more prone to suffer from AIDS-related and non-AIDS-related complications and death [36,41-45,51-53]. These patients (the proportion ranges between 15-40% depending on

the study) are referred to as immunological discordant or incomplete responders, although there is no agreement on the definition of immunological response failure and several definitions are used in several reports [reviewed in 54]. For example, a reasonable definition taking into account a shorter period of time under successfully HAART, could be a rise in $CD4^+$ T cells lower than 100 cells/mm^3 after two years of treatment (i.e. less than half of the expected recovery) [28]. The failure in the immune recovery can be explained by insufficient production or by excessive destruction of $CD4^+$ T cells due to several, multiple, concomitant, and overlapping conditions [41,42,47,52,55,56, reviewed in 28,51,54]:

1. The irreversible damage (fibrosis) caused by HIV infection of lymphoid organs might lead to the failure of the bone marrow to produce hematopoietic stem cell precursors for different lineages of blood cells (including the lymphoid progenitor cells), and to an impaired thymic function (resulting in a deficient new T cells differentiation and export to the periphery). Moreover, local fibrosis in lymph nodes and GALT may alter survival and proliferation of $CD4^+$ T cells;
2. An advanced age is linked to a reduced thymus output, to a higher immune activation, and to age-associated changes in the immune system (immunosenescence);
3. The HIV infection seems to accelerate the aging process causing immunosenescence that could be in part irreversible and contribute to immune activation;
4. Men have been shown to present lower thymus output than female; this has been associated to the anti-apoptotic effect of female sex steroids;
5. Residual viral replication may occur in some cells of some apparently virological suppressed patients accounting for ongoing cytopathogenicity of the virus and immune activation;
6. Presence of more cytopathogenic virus as those that use CXCR4 as a co-receptor;
7. Some antiretroviral agents like zidovudine, that is toxic for haematopoietic progenitor cells, have been associated with worse reconstitution; on the contrary, some antiretroviral agents like raltegravir and the CCR5 antagonist maraviroc have been linked to a favorable recovery (the high accessibility of raltegravir to anatomic compartments could efficiently inhibit the residual HIV replication [57] and perhaps maraviroc, due to the fact that CCR5 is now recognized as a co-activation molecule, could decrease immune activation, though the beneficial role of maraviroc was not verified by all [58]);

8. Although T cell activation declines with virologic suppression, it is not abolished and may persist elevated in some patients continuing to drive apoptosis;
9. Enhanced T cell activation and trapping in secondary lymphoid organs (due to high-expression of adhesion molecules) has been reported;
10. Some genetic polymorphisms account to an increased susceptibility to activation or apoptosis, or to an increased production of pro-inflammatory cytokines;
11. Relative deficiency in interleukin (IL) -7 (a key cytokine with a positive effect on thymopoiesis, a survival factor for $CD4^+$ and $CD8^+$ T cells, and promoting homeostatic proliferation of peripheral T cells in lymphopenic conditions) production or function, or reduction in IL-7 receptor expression on $CD4^+$ T cells (administration of recombinant human (rh) IL-7 to human has been promising in preclinical trials with increases in $CD4^+$ T cell counts only after a short duration of treatment [59]);
12. Microbial translocation due to a leaker immune barrier may be one of the drivers of immune activation;
13. Co-infections by hepatitis C virus (HCV), cytomegalovirus, other herpesvirus, or even by *Mycobacterium avium complex,* could account for an increased immune activation.

A low $CD4^+$ T cell count at the therapy onset has consistently been shown to be a good predictor for an immunological non-response as in these situations, several of the above mentioned conditions are present, among others. An advanced stage of the disease may cause a more advanced fibrosis of lymphoid organs, a more intense microbial translocation, the development of other co-infections, a more advanced immunosenescence, the emergence of more aggressive X4 strains, and therefore, a more intense immune activation.

The role of regulatory T cells (Treg) in immune restoration has been debated and while some authors refer to them as beneficial, as they diminish immune activation [60], others, however, report a harmful role of those cells [53,56,61] perhaps due to the fact that they impair specific anti-HIV immune response (via both indirect, by secreting immunosuppressive cytokines, and direct, by driving apoptosis of immune cells), they inhibit lymphopenia-induced proliferation [61,62], they may drive a more intense microbial translocation (their increase in mucosal barrier hampers the Th17 response against several microbes) [63,64] or they may account for exacerbated fibrosis of lymphoid tissues prejudicing immune reconstitution (by increasing levels of TGF-β1 and hence exacerbating collagen deposition) [65].

Advances made until present-day in the HIV field are a result of science, advocacy, political commitment, and effective partnerships with affected communities. Recently, the report of cases of sterilizing cure (elimination of all HIV-infected cells as it occurred in the Berlin patient) [66], functional cure (long-term control of HIV replication after HAART suspension as it seemed to be achieved in the Mississippi baby [67] or in 14 patients in the VISCONTI cohort [68]), and other cases of sustained reduction in HIV-reservoirs [69], had revived the hopefulness in a future free of AIDS. Several HIV-cure-related trials are being performed worldwide and although one is in the commencement, many now believe there may be a step forward in discovering a cure. The optimism persists despite the recently disappointing turn of events for the Mississippi baby (the child was found to have detectable HIV levels in the blood during a routine clinical care visit on July 2014).

Until a cure is achieved, clinicians have to diagnose early and establish the most appropriate antiretroviral treatment for each patient and observed them clinically and analytically for a sustained viral suppression and consequently, a good immune recovery is reached, minimizing as much as possible what can hinder this recovery. It is worthwhile to mention that effective antiretroviral therapy, although allowing immune recovery, preventing AIDS-related complications, and prolonging half-life with better quality of life, it does not fully restore health. As mentioned above, immunosenescence, residual persistent immune activation/inflammation, and also, some HAART toxicity and prevalence of traditional behavior risk factors (as tobacco or intravenous drug use per example), account for a number of non-AIDS-related co-morbidities [26].

Patients should then be encouraged to maintain a proper medication adherence and a healthy life in an attempt to prevent the onset of co-morbid conditions which if appear, should be promptly diagnosed and treated. Another major challenge is to maintain access to and funding for lifelong HAART to the more than 35 million of people with HIV infection worldwide. Efforts must be done to reach that aim at manageable costs to health systems.

Since the incomplete immune recovery under effective long-standing HAART carries long-term risks of disease progression and death, and there are no efficient therapeutic strategies currently in these situations, is of vital importance to find solutions. Scientists, apart from trying to discover a sterilizing or at least, a functional cure, have to investigate the immune-virological pathways behind insufficient immune recovery providing tools to anticipate it, and/or novel therapeutic strategies to treat it. One of the possible pathways involved and still insufficiently

explored is one that concerns the Treg. If further research in this field namely functional and longitudinal analyses of Treg in HIV infection, will demonstrate a well defined and sustained role of this $CD4^+$ T cell subset, immunotherapeutic manipulation or intervention involving Treg-cell number and/or function [70] could be attempted in HIV-infected patients already under treatment aiming to improve immune restoration. In the next session the role of Treg on HIV-infection and immune restoration will be detailed.

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1.2. Human Regulatory $CD4^+$ T Cells (Treg)

1.2.1. Introduction

One of the supreme tasks of our immune system is immunological tolerance i.e. the ability to distinguish self from non-self, offering protection to our own cells while fighting potential pathogens through the recognition of strange incoming antigens. Immunological tolerance has two components, a central and a peripheral one. Central tolerance comprises the processes of positive and negative selection that take place in the thymus where, presumably, only T cells with moderate affinity to self-peptides presented by self MHC molecules fully differentiate (positive selection) and leave thymus. On the other hand negative selection is responsible to eliminate differentiating T cells that show too much affinity/avidity to self-peptides presented by self-MHC molecules. However, thymic negative selection is known to be insufficient to avoid the differentiation of some T cells that react too strongly towards self-peptides-MHC complexes. In the periphery, Treg, a specific subset of T cells, play a very important role in restraining those auto-reactive T cells [1].

The existence of a particular subset of T cells playing a negative immune regulation role has been a great controversy among immunologists for many years [2]. In the early seventies, Richard Gershon had indorsed the predecessors of these cells, the suppressor cells [3], but that field faced a major setback and had been mostly abandoned by the majority of the scientific communities after the negative results found using more modern tools for immunological investigation [reviewed in 2]. However, in the mid-1990s, immunologists recover their enthusiasm about T cells that had as a main function controlling/reducing the activity of other T cells - Simon Sakaguchi and his colleagues rediscovered what are now named Treg [4] - the discovery that some spontaneous diseases in mice and humans were the consequence of the lack of Treg [4-6] has rendered these cells definitely accepted. Changes in their number or function were linked to many events, but in particular to autoimmunity [7], cancers [8], allergies [9], and the outcome of infectious diseases [10-12] in particularly some chronic infections [11].

Treg are essential for the maintenance of the immune system's equilibrium; playing key roles in the maintenance of immunological tolerance, preventing autoimmunity, and limiting chronic inflammatory diseases [7]. Moreover, they can be induced to inhibit transplant rejection reactions, and can limit immune-mediated tissue damages in infections [8,11,12,13]. However,

they also suppress antitumor immunity and may prevent sterilizing immunity against certain chronic infections [8,11,12,13].

The infection by the HIV leads to a deep imbalance of the immune system and growing evidence suggests that Treg may influence the outcome of this infection.

Human Treg represent a minority of T cells comprising for most individuals less than 10% of the blood $CD4^+$ T cells [13,14], and it seems likely that manipulating the number and function of these cells will one day be a useful medical procedure in several diseases [12].

This section will detail information on Treg namely their origin and main characteristics, their action mechanisms, and their role in infectious diseases focusing mainly on the interrelationship with HIV infection.

1.2.2. Where do Treg come from?

Treg can be from thymic origin (named as naturally occurring Treg - nTreg), or be induced in the periphery from conventional $CD4^+$ T cells (designated as induced, adaptive or converted Treg - iTreg) [reviewed in 11,12,15-17] (Figure 1 [17]).

Treg from thymic origin - naturally occurring Treg - nTreg

The thymus is essential for the differentiation and proliferation of lymphocytes precursors into mature T cells, setting up the peripheral T cell pool before birth and early in life. The thymus undergoes an age-dependent involution associated with a decline in function and consequently, in thymopoiesis. However, it is now generally accepted that thymopoiesis can be maintained even up to the eighth decade of life, or reactivated under particular circumstances such as after a massive exhaustion of the T cell pool as following haematopoietic stem cell transplantation [18,19].

In the thymus, T-cell precursors undergo recombination of sequences within its antigen-binding T-cell receptor (TCR) genes to generate a TCR unique to that cell, expressed on their surface. A broad diverse TCR repertoire is thereby generated. As the epithelial cells in the thymus have the unique capacity to express most of the human genome, the differentiating T cells can be exposed to the normal repertoire of human proteins [20]. T cells expressing nonfunctional TCRs, TCRs that cannot interact with self-MHC molecules, or those that react too strongly with MHC expressing self-peptides, are eliminated (negative selection) [21,22]. The selection process leads

to the generation of MHC class I-restricted cytotoxic $CD8^+$ T cells and MHC class II-restricted helper $CD4^+$ T cells that are for the great majority self-restricted and self-tolerant. T cells exiting the thymus are referred in the periphery as naive recent thymic emigrants (RTE). These cells express high levels of CD-31 on their surface [23] together with CD45RA.

Treg whose development program is still unclear, may possibly be an independent thymus-derived T cell lineage which commitment may occur earlier than the mature CD4 single positive stage [24,25]. How the thymic selection works, deleting self-reactive T cells but also giving rise to Treg specific for self-antigens, remains to be elucidated [22]. Avidity of the TCRs to these self-antigens-MHC complexes seems to determine the fate of T cell; whereas an intermediate affinity TCR to self-antigens would lead to a conventional T cell, a relatively high self-reactive TCR (but not so much as to be deleted) would originate Treg (a transient expression of a functional IL-7 receptor and an increased level of the anti-apoptotic Bcl-2 could protect them from negative selection [24]).

Naturally occurring Treg can be activated and expanded by TCR stimulation and by other means [reviewed in 11]. These cells have a diverse TCR repertoire, but they are mainly self-reactive helping to prevent autoimmunity. However, in an inflammatory response, the released of tissue antigens may activate these cells and also maybe due to a possible overlap between the TCRs of Treg and non-Treg, they can sometimes react specifically with microbial antigens [11]. Alternatives to TCR stimulation, although the exact mechanism is controversial and not fully elucidated, may include: 1) stimulation of other receptors either in the nTreg themselves or in intermediary cells of the immune system which will then activate Treg: nTreg seem to express pathogen recognition receptors (PRRs) like toll-like receptors (TLRs) and others that can be triggered by molecules expressed by microbial agents (pathogen-associated molecular patterns - PAMPs) [11 and references therein], or by reaction products produced by the host and released after infection or inflammation such as galectins (galectin-1 and galectin-9) and cellular metabolites (retinoic acid - RA, and others) [26]; or 2) stimulation by cytokines generated in the microenvironment of an infection like Interleukin (IL) -2, transforming growth factor-β (TGF-β), or tumor necrosis factor-α (TNF-α) [11 and references therein].

Treg induced in the periphery from conventional CD4+ T cells - induced, adaptive or converted Treg - iTreg

The iTreg are derived, in the peripheral lymphoid organs (lymph nodes, spleen, and mucosa-associated lymphoid tissue - MALT), from conventional CD4+ T cells [27]. There is an induction of the expression of suppressor surface markers after TCR stimulation mainly by non-self antigens (sub-immunogenic doses of peptides) in an appropriate environment of cytokines (TGF-β, IL-10 and IL-2 are of particular interest [28-32], and possibly IL-35 [33]), RA [30,31], and co-stimulation. Some dendritic cells (DCs), in some body's compartments and in certain circumstances, are able to produce TGF-β or IL-10, and so, potentially help conventional T cells to convert into Treg [31,32]. Also, the anti-inflammatory molecule thrombospondin (TSP-1) secreted mainly by platelets and antigen presenting cells (APCs), interacts with CD47 receptor on CD4+ T cells promoting their conversion on Treg, as IL-10 and TGF-β seem to do [34]. iTreg population seems to be of central importance, for example, to maintain a non-inflammatory environment in the gut, suppressing immune responses to commensal microorganism-derived antigens, and environment and food allergens [12,17].

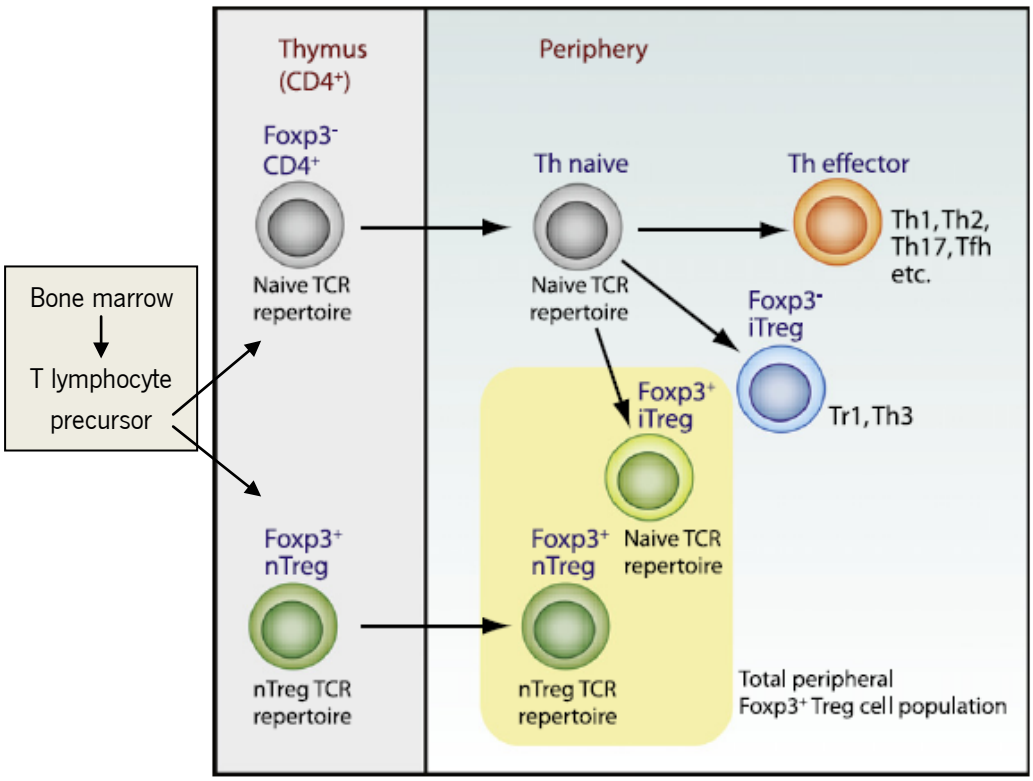

Figure 1. Thymic and peripheral generation of Treg cells. nTreg – naturally occurring Treg, iTreg - induced, adaptive or converted Treg, Th1, Th2, Th17, Tfh - CD4+ T cells subsets, FOXP3+ - expression of the master control gene forkhead-box nuclear transcription factor P3, TCR - T-cell receptor, Tr1, Th3 – other FOXP3- iTreg subsets (adapted from [17]).

IL-2 appears to have an important role in Treg's (both nTreg and iTreg) initial production, and subsequent maintenance in the periphery (due to peripheral expansion and altered death rate of existing cells) although its real role in Treg differentiation remains under debate [21,35]. Experiences with mouse models demonstrated that this cytokine or its receptor (α chain of IL-2 receptor or the CD25 molecule highly expressed on Treg' surface) deficiency resulted in severe deficiency of Treg [35]. Additionally, most recently, it has been demonstrated that the large proportion of the total $CD4^+$ cells expanded due to IL-2 treatment, in patients infected by HIV, shared phenotypic, functional and molecular characteristics with Treg [36].

As well as conventional T cells can be converted into Treg, the opposite seems also to be possible as Treg are highly prone to functional plasticity [37-40]. Recent findings suggest that T helper cell differentiation is more plastic than previously appreciated and lineage reprogramming can occur. Each $CD4^+$ T cell subset can adopt alternate cytokine profiles in response to cytokine environmental changes and to inducible expression of key transcription factors [37-40]. Among the T cells subsets, Treg and Th17 cells have been described as displaying the highest propensity to switch to other phenotypes [38].

Under appropriate conditions, the function and phenotype of some subpopulations of Treg can change, losing their inhibitory effects and even becoming pro-inflammatory participants. The environment that could cause such plasticity, namely one rich in pro-inflammatory cytokines and Treg-attracting chemokines, is common in many virus induced inflammatory lesions [11,38 and references therein]. Thymus-derived nTreg cells and peripherally derived iTreg cells appear to differ in their propensity for reprogramming, being the last ones more prone to change their function [38]. While TGF-β is required for the differentiation of iTreg and for the maintenance of nTreg after their egress from thymus, it is also needed for the development of Th17 (given that IL-1β and IL-6 are also present) and may be a relevant link between the two T cells lineages.

1.2.3. How are Treg Characterized?

The identification of Treg has been hampered for years due to the lack of specific markers. Treg are $CD4^+$ T cells and share many surface marker characteristics with activated $CD4^+$ T cells. Treg were first identified on the basis of their high level of CD25 expression [14]. Subsequently, they were identified by the expression of the master control gene forkhead-box nuclear transcription factor P3 (FOXP3), a key regulatory gene for the development and function

of Treg [41]. Further human studies, however, demonstrated that activated $CD4^+$ T cells may also up-regulate the expression of CD25 and can transiently express FOXP3 [42]. More recently, it was shown that Treg express low levels of CD127 (the α chain of IL-7 receptor, negatively regulated by FOXP3), and therefore this surface molecule is considered useful as an additional marker to identify this population [43,44]. Lately, another surface marker specific for nTreg emerged - the orphan receptor glycoprotein A repetitions predominant (GARP or LRRC32) [45,46] – a transmembrane protein that seems to be selectively expressed by Treg and mainly by activated human Treg, but not by activated effector T cells. Moreover, the GARP seems not only to identify activated Treg but also discriminates those with the highest suppressive activity [45-47]. Thus, GARP could play an important role on the identification of functionally active Treg.

Even though the utility of other putative Treg markers in mice or in humans (as the cytotoxic T-lymphocyte associated antigen 4 - CTLA-4, glucocorticoid-induced TNFR-related protein – GITR) [30], is still debated, $CD3^+$ $CD4^+$ $CD127^{low}$ $CD25^{high}$ $FOXP3^+$ currently represent the most reliable markers set to identify this T cell subset.

Recent studies have also shown that the expression of CD45RA or CD45RO, which are mutually exclusive, are particularly useful markers when combined with CD25 and/or FOXP3 enabling the identification of functionally and phenotypically diverse Treg subsets [30,48-50]. Miyara *et al* [48] have defined, based on the expression of CD45RA/RO, three Treg subsets; resting Treg cells ($CD45RA^+FOXP3^{low}$), activated Treg cells ($CD45RA^-FOXP3^{high}$), both of which are suppressive *in vitro* (although *in vivo* activated Treg seem to be the main effectors of suppression [30]), and cytokine-secreting non-suppressive T cells ($CD45RA^-FOXP3^{low}$).

The combination of CD45RA and the homing lymph tissue marker (CCR7) allows the classification of T cells in three populations: naïve ($CD45RA^+CCR7^+$), central memory ($CD45RA^-CCR7^+$) and effector memory subsets ($CD45RA^-CCR7^-$) [51]. Furthermore, the combination of CD45RA and human leukocyte antigen (HLA)-DR may be used to define effector Treg ($CD45RA^-HLA\text{-}DR^-$) and terminal effector Treg ($CD45RA^-HLA\text{-}DR^+$) [30].

Others Treg subsets, like Tr1 and Th3, and the more recently identified - iTreg35 - seem to be iTreg converted from non-regulatory T cells under particular conditions, expressing some of the Treg surface markers (although, probably do not express FOXP3) and sharing some of the suppressive properties of Treg as IL-10, TGF-β or IL-35 secretion [15,33,52,53].

1.2.4. How do Treg Work?

After activation, Treg are able to modulate the activities of a wide variety of cellular components of both innate and adaptative immune responses, reducing the magnitude of the protective T cell response and preventing inappropriate or exaggerated immune activation of T cells induced by pathogens. Furthermore, these cells down-regulate self-reactive T cells promoting tolerance and avoiding autoimmunity. Moreover, during spontaneous lymphopenia-induced proliferation, Treg faired to be a limitative factor to the disparity of rate proliferation between varied T cell clones, and therefore lead to preservation of the TCR repertoire diversity and to protection against the development of inflammatory pathology and autoimmunity [54].

Suppressive activities of Treg include a reduction in the magnitude of the antigen-specific $CD4^+$ and $CD8^+$ T cell responses by a suppression of activation, proliferation, differentiation and function of those cells [55], as well as an inhibitory effect on cell trafficking of activates antigen-specific T cells to infected sites [56]. Inhibitory effects on APCs, NK, NKT, B and mast cells function as well as inhibition of the pro-inflammatory activity of macrophages and neutrophils are others mechanisms used by Treg to control immune response [57,58].

The mechanisms used by Treg to exert suppressive function are still unclear. Although, studies *in vitro* and making use of animals models have shown multiple mechanisms that could be used by Treg to suppress their counterparts, there is still no absolute certainty if all of them are implicated *in vivo*, and if so in animals models, if they also happen in humans. At least three mechanisms of action of these cells [reviewed in 29,30,57,59-61] have been described and one of them, the cell-to-cell contact, seems to be the most important (in Figure 2 are shown some suppressive mechanisms of Treg [61]):

1) By cell-to-cell contact, Treg can cause the death or the suppression of responder T cells (Figure 2A). The cell death can occur by direct lysis by a perforin/granzyme-dependent mechanism, or also by induction of apoptosis through Fas ligand-Fas interactions (human Treg have been found to express Fas and FasL). Also, Treg were found to induce death in B cells, monocytes and DCs. In addition to causing death of other immune cells, Treg might suppress immunological activities through: 1) release of cyclic adenosine monophosphate (cAMP) via gap junctions (leads to inhibition of T cell proliferation and IL-2 production); 2) the deliverance of a negative signal through surface molecules on Treg (CTLA-4 seems to be the most important); and 3) generation of pericellular adenosine, catalyzed by CD39 and CD73 in Treg, that via A2A

receptor promote the sequential increase in intracellular cAMP. Treg with upregulation of CD39 or CD73 expression may have a more potent suppressive effect [62,63]. The binding of Treg CTLA-4 to CD80/CD86 on the surface of the DCs also indirectly suppress T cell response: via modulation of APC function (down-modulating of CD80/CD86 on DCs, outcompeting the activating receptor CD28 on T effector cells, and inhibiting DC maturation) and inducing APCs to express the enzyme indoleamine 2,3-dioxygenase (IDO). IDO catabolizes conversion of tryptophan to kynurenine, a toxic product to the activated T cells (and privating effector T cells of tryptophan, an essential amino acid for their survival [12]) (Figure 2B). Apart from CTLA-4, several other molecules are reported to contribute to the suppressive function of Treg via direct Treg – T effector cells interactions and/or via modulation of APC function like (Figure 2A e 2B): lymphocyte activation gene 3 (Lag-3 [64,65]), Ig-like type 1 transmembrane protein (CD83), lymphocyte function-associated antigen 1 (LFA-1 or CD11a-CD18), GITR (tumor necrosis factor receptor superfamily or TNFRSF18), herpesvirus entry mediator (HVEM or TNFRSF14) [66], or programmed death-1 (PD-1 or CD279).

2) Production of suppressive soluble factors such as the cytokines TGF-β, IL-10 and IL-35 (Figure 2A). TGF-β may act as a mediator of suppression as a membrane-bound form (although this is still controversial), may condition responder T cells to be receptive to suppression, may maintain FOXP3 expression and, as mentioned above, may additionally contribute to the differentiation of other T cells into Treg-like cells. Suppression via IL-10 production has been shown to be a potent immunoregulatory mechanism with anti-inflammatory functions in *in vivo* models of Treg-controlled inflammation and homeostatic expansion [67]. IL-35 not only acts as an effector cytokine of Treg, but also appears to regulate Treg homeostasis (it was described to expand Treg defined as $CD4^+CD25^+$, and to induce a novel T cell population with IL-35-dependent regulatory function [33]).

3) Competition for IL-2, and possibly other growth factors, inducing cytokine deprivation-mediated apoptosis in responder T effector cells (Figure 2A). In addition, Treg seem to compete by biochemical factors, for example, cystine, interfering with its DC secretion to T effector cells, indirectly depleting these cells of a factor strongly related with a proliferative and functional response [61] (Figure 2B).

It is conceivable that the main suppressive pathway involved in Treg action depends on the particular disease or compartment involved, or that these multiple suppressive mechanisms operate simultaneously and synergistically, not being sufficient the disruption of one of them to

impair Treg mediated immune suppression [29]. Nonetheless, CTLA-4-dependent and possibly an IL-2-dependent mechanism seem to be the central suppressive mechanisms [29].

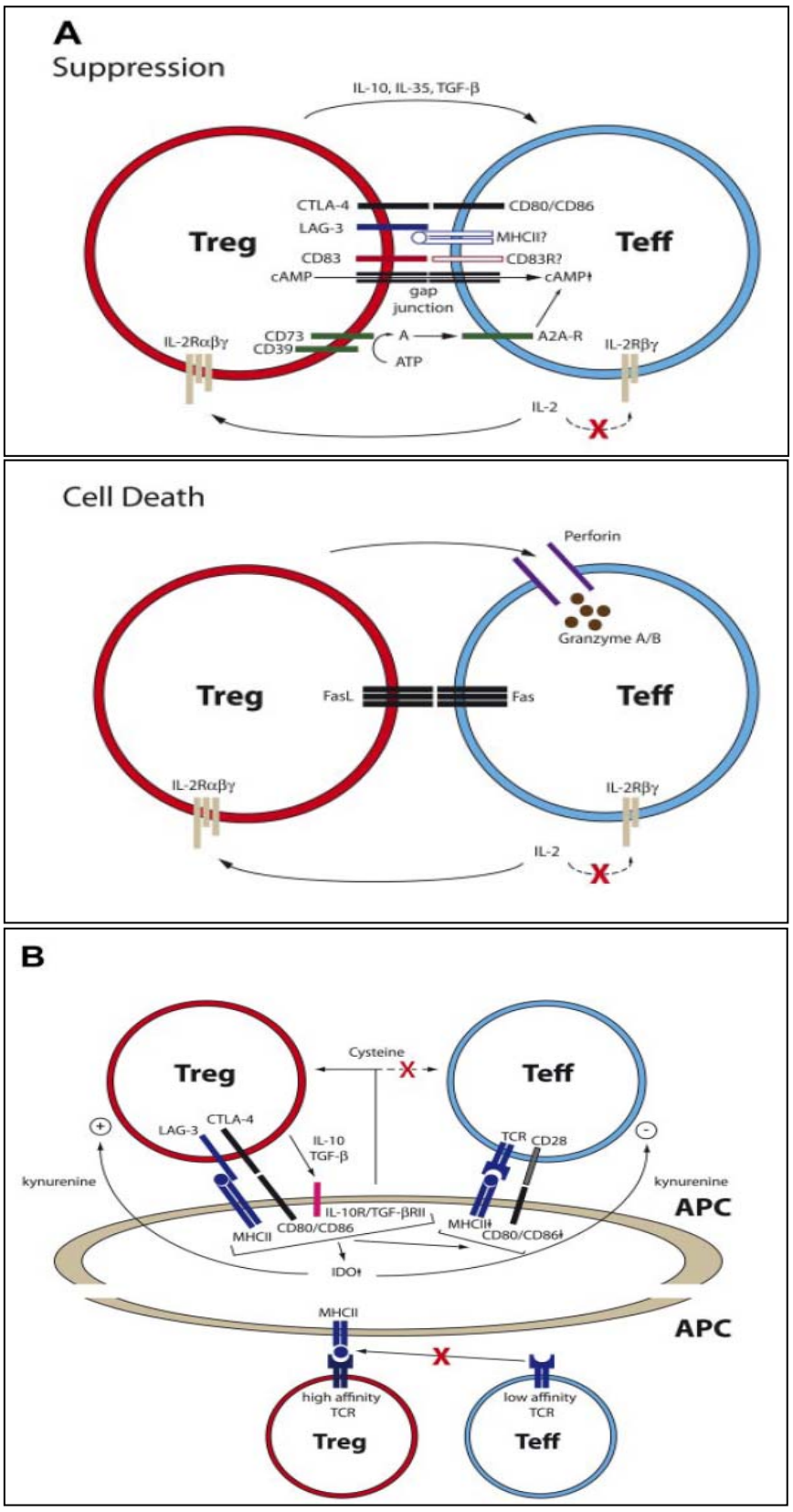

Figure 2. Scheme of suppressive mechanisms of naturally occurring Treg. A) Direct suppression and induction of apoptosis. B) Modulation of Teffector responses via APCs (extracted from [61]).

1.2.5. What is the Role of Treg in Infection?

The study of Treg in human diseases has increased over the past three decades. There are countless papers on the possibility of these cells to become a therapeutic option in almost any situation where the suppression of an exaggerated/exacerbated immune response may be relevant; transplantation, autoimmunity, allergies, cancer, and infectious diseases [59 and references therein].

Leaning mainly on infectious diseases; for the control of immune responses to infection [10-12], several mechanisms are triggered, and accumulating evidence suggests an important role of Treg in controlling these responses. The nature of the Treg response in an infection can influence the disease's pathogenesis and outcome [10-12]. There are instances where Treg expand in response to an infection by a pathogenic agent leading to an inadequately protective immunity, and contributing for the dissemination and/or persistence of the agent. In mice, for example, infection by Herpes simplex virus (HSV) has been shown to lead to Treg expansion, which delays the recruitment of protective $CD8^+$ T cells to the infected mucosal, facilitating the infection of central nervous system [66,68]. Studies also suggest an important role for Treg in establishing chronic infection by HCV [69-72], and by multiple other pathogens (virus, bacteria, parasite, and fungus) [10,69,72]. Contrariwise, if the pathogen agent inhibits Treg function, that could allow a vigorous $CD8^+$ T cell response which contributes for the establishment of an effective clearance of the pathogen (perhaps explaining why chronicity is uncommon in hepatitis A infection [73]). Sometimes, however, while the presence of Treg facilitates the pathogen persistence, that can be useful once the concomitant immunity developed protects the host from reinfection [74]. Moreover, the Treg expansion can be beneficial, limiting immune-mediated inflammatory conditions, and therefore restraining immune-mediated tissue damages. [75]. Indeed, some pathogens lack cytopathogenicity, and tissue damage is mostly due to T-cell-mediated response to the infection. Controlling the extent of such tissue damage appears to be a major function of Treg. In an infection's environment as pulmonary infection by Respiratory Syncytial virus, Treg also showed to influence the timely trafficking of other immune cells to the infection site and hence control both the growth and spread of infection and the immunopathology during the acute pulmonary virus infection [76]. Moreover, some infectious agents modulate Treg trafficking to the infection's environment through the stimulation of

receptors in their surface that are important homing molecules (CD103 expression on Treg and skin infection by *Leishmania major* [77]).

Concerning chronic and persistent infections, it appears that Treg could play even a more consequential role influencing outcome (comparing with acute infections). One human virus, HIV, has received the most attention, as it is a major cause of illness and death, and lacks effective prophylactic or curative therapy.

1.2.6. What is the Role of Treg in HIV Infection?

Treg, as important immunomodulatory cells, have been investigated as potentially relevant in the outcome of HIV infection. Although a great number of papers have been written about Treg and HIV infection, results continue to be conflicting. Deciphering the exact role of Treg in HIV pathogenesis is of crucial importance to fully understand HIV induced immune suppression as well as immune reconstitution. In addition, understanding how Treg influence the immune system among HIV-infected patients may contribute to the determination of potential new strategies to modulate the disease.

Concerning HIV infection, different aspects have been investigated, namely: 1) whether or not Treg are directly infected and altered by the virus; 2) to what extent they are depleted/expanded; 3) if their function is preserved; and 4) what is their role during the HIV transmission, the course of disease progression from HIV infection to AIDS, and in the immune reconstitution under HAART.

In relation to the direct infection of Treg by HIV, it has been reported that Treg cells are susceptible to HIV infection (these cells express, in addition to CD4, CC chemokine receptor type 5 - CCR5 and the CXC chemokine receptor type 4 - CXCR4) [78-80]. However, it remains to be clarified to what extent Treg are infected, which Treg subsets are preferentially infected and the effects of the infection by HIV in Treg [78-83]. Some authors suggest that Treg are highly susceptible to HIV infection and are killed by viral replication [78]. Other studies, however, argue against the increased cell death of HIV-infected Treg [84,85]. In another study, the transcription factor FOXP3, critical for Treg development and function, was shown to modulate the HIV promoter's transcription activity [82]. Thus, permissiveness to HIV replication in Treg may be reduced, partly as a result of FOXP3-mediated inhibition of HIV transcription. Others, however, suggest that FOXP3 may also facilitate HIV transcription by inhibiting histone deacetylases

(HDACs) rendering the proviral form more accessible for efficient transcription [83]. Interestingly, although using non-infectious virus with an intact envelope, a study showed that exposure of Treg to HIV selectively promotes their survival and expansion via a CD4-gp120–dependent pathway (the CD4-gp120 interaction resulted in the inhibition of Treg apoptosis) [81].

In respect to the level of Treg depletion/expantion in HIV-infected patients, it has been reported to vary with several factors namely disease's phase. Also, some disagreement exists on the reported evaluation of Treg role on the evolution from HIV infection to AIDS as well during immune reconstitution during HAART. Part of these contradictory data may result from the heterogeneity of the cohorts analyzed, from the different gating strategy and/or different set of Treg markers used to define Treg, and/or from the use of FOXP3 mRNA measured by reverse transcription polymerase chain reaction (RT-PCR) in T cells to define Treg instead of its expression by flow cytometry.

During primary HIV infection, a decrease in Treg percentage was observed in the blood [86] reflecting, according to the authors, a susceptibility of these cells to HIV infection [78,79] or their recruitment to inflamed sites (lymphoid tissue) [81,84]. Nevertheless, the opposite (an increase in Treg frequency during the acute phase of the HIV-infection) was also reported by others [87], although in this last study, a lower number of patients were analyzed and a more incomplete set of Treg markers was used.

After the acute phase, during the chronic phase without treatment, although both absolute numbers of peripheral Treg and proportions of Treg among total $CD3^+$ T cells are mainly found to be decreased in patients comparing to healthy donors, most studies show that the proportion of peripheral Treg among $CD4^+$ T cells expand - the numbers of Treg decrease progressively over time but at a lower rate than the whole $CD4^+$ T cell population [86-90 reviewed in 91,92, and others studies whose references are therein]. Thus, most studies argue for a general increase in Treg frequency although this is not reported by all published studies [93,94]. The increased Treg representation could be the consequence of reduced HIV-mediated destruction comparatively to other $CD4^+$ T cells [81], enhanced generation by thymus [95], conversion of uncommitted cells to become Treg (in HIV-infected individuals, DCs seem to be more prone to induce Treg [96,97], and some local cells are induced to produce TGF-β [81,93,98]), higher survival (relative resistance of Treg to activation-induced cell death), or increased proliferation (increased expression of Ki67, a marker of the cell cycle, was found in circulating Treg from untreated, chronically infected patients) [89,92,99]. Finally, several studies

suggest the presence of Treg responding to HIV proteins, and therefore expanding as a direct consequence of HIV infection [81,90,100].

Successfully HAART treatment seems to result in a decrease of Treg frequencies (even if a transient increase in the first months after HAART occurs [89,101]) according to most [51,84,88,89,101-106], but not all [87,90,107] studies. Unsuccessfully treated or untreated patients, in contrast, often retain high Treg frequencies [106].

If the rise in Treg frequency frequently observed in HIV-infected patients represents a concomitant increase of a suppressor subset remains to be clarified. Several reports suggest that Treg function is preserved in HIV infection and may even, in same stages of the disease, be increased [80,81,87,90-92]. It has been reported that Treg with upregulation of $CD39^+$ expression (Treg with more potent suppressive effect [62,63]) are expanded in HIV infection [88,89,108]. Furthermore, some authors demonstrated an increased sensitivity of effector cells to Treg-mediated suppression, in infection by HIV [108,109]. It has also been suggested that HIV *tat* could increase CTLA-4 expression and in addition the suppressive potency of Treg [110]. However, recently, the reports by Pion *et al.* [111] and by Angin *et al.* [112], have described *in vitro* that HIV-infected Treg show impaired suppressive capacity and a decreased expression of genes critical to Treg function. In some instances, the suppressive capacity of Treg may also be impaired as it seems to happen in the immune reconstitution inflammatory syndrome (IRIS). IRIS is a complication that occurs in a small percentage of patients that initiate antiretroviral therapy at a stage of advanced disease. It is characterized by an exaggerated and/or inappropriate immune system reaction to a subclinical undiagnosed (unmasking IRIS) or to an already diagnosed and under correct treatment (paradoxal IRIS) infection. Although, immunopathogenesis of IRIS remains unclear, a dysfunction of Treg (even with a Treg expansion) has been proposed by some authors as a potential mechanism responsible for a deregulated $CD4^+$ T and/or NK cell response and consequently, on that exacerbated pro-inflammatory immune response [102]. Others argue that the Treg dysfunction during IRIS is due to a rapid rebound of conventional T cells after HAART initiation, not followed by a comparable rise of Treg, leading to a rapid decrease in the frequency of Treg and to an exacerbated immune response to pathogens and hence to IRIS [51].

Several roles have been assigned to Treg during HIV infection - a great amount of controversy exists and both detrimental and beneficial roles of Treg are suggested. Detrimental

effects of Treg could result from an excessive activity of Treg weakening HIV-specific $CD4^+$ and $CD8^+$ T cell responses [86,90,93,113]; a beneficial effect could be associated with the ability of Treg to minimize the extension of T cell activation controlling the availability of HIV targets and preventing immune-based pathologies [114-116]. It remains to be determined what of these contradictory main effects plays a greater role in the disease. Other effects have been suggested; a detrimental facet of Treg could be their eventual involvement in the exacerbated collagen deposition within T cell zones, shown in simian immunodeficiency virus (SIV) infected simian, that could destroy the lymphatic tissue architecture and hamper $CD4^+$ T cells to reach essential sites or $CD4^+$ T cells reconstitution in infected patients (the pathway involved could be the increased level of TGF-β produced by Treg) [85]. Also, the ability of Treg to hamper the lymphopenia-induced proliferation could be one of the mechanisms involved in the poor immune reconstitution reported by some authors [54,117]. It appears that the different roles of Treg described above, could be occurring at different times and with different intensity during the distinct phases of the disease: 1) transmission of the virus, 2) disease progression, and 3) response to HAART:

- During the inter-individual transmission of the virus, a high Treg frequency could be protective by reducing T cell activation, and consequently decreasing the pool of susceptible $CD4^+$ T cells (as have been observed in HIV-exposed seronegative women and in HIV-exposed uninfected infants or neonates) [114,118]. Additionally, in untreated acute SIV infection, the use of a CTLA-4 blockade procedure (to block Treg function) increased viral replication and $CD4^+$ T cell loss, particularly at mucosal sites (by providing more target cells for the virus by decreasing the threshold for T-cell activation) [115]).

- During the HIV infection progression without treatment (the chronic infection phase) some observations are consistent with Treg playing a detrimental role, or that high levels of Treg are, at least, not an advantage for a good evolution of the disease: 1) though not consistently found, most studies showed an inverse relationship between Treg frequency and $CD4^+$ T cell counts (Treg frequency and plasma viral load were either not or positively correlated) [87,89,107,108,117]; 2) long-term non-progressors (HIV-infected individuals who maintain high $CD4^+$ T cell counts even in the presence of HIV replication in the absence of therapy) and elite controllers (HIV-infected individuals who maintain undetectable viral loads in the absence of any treatment) seem to have lower levels of Treg frequency in peripheral blood and/or rectal mucosa than other infected individuals (these cell numbers may be similar to [87,88] or even

lower than those found in healthy individuals [119,120]), and in addition, these patients seem to have a polymorphism which does not allow their Treg to efficiently suppress HIV-specific proliferative responses of $CD8^+$ T cell [121]; 3) furthermore, some studies showed that extensive Treg accumulation within the lymph nodes was associated with a chronic progressive HIV and SIV infection [81,93,122].

The idea that Treg may play a positive role in the context of HIV infection, by limiting exaggerated immune activation, which is a prelude to the onset of AIDS, have also been reported. But if that were to be the case, it would be expected that a high Treg frequency would be related to a lower immune activation. Several studies investigated the potential ability of Treg to control exaggerated immune activation/inflammation in the context of HIV infection without treatment. Even though Treg may protect the host against immune-mediated damage (one study showed that Treg attenuate HIV-associated neurodegeneration [123]), most studies performed in untreated chronically infected patients, or in those interrupting HAART, showed a positive relationship between Treg frequencies and T-cell activation regardless of the activation markers analyzed (i.e. CD38 and/or HLA-DR or CD69 expressed on CD8 and/or $CD4^+$ T cells) [87,88,107,117,124]. Furthermore, rectal Treg frequency was also found to positively correlate with rectal T-cell activation in untreated patients (a higher Treg frequency there, could be responsible for a lower Th17 response and lower specific response to several microorganisms and hence, a higher microbial translocation) [119,125]. Thus, Treg seem to be inefficient to control high immune activation in viremic patients. However, it could be that, for example concerning the beneficial effect of Treg decreasing immune activation, the most important would be absolute numbers (found to be decreased in these patients) instead of Treg percentages and some studies point into that direction as exploring the relation between Treg measured by absolute numbers and immune activation and found a negative relation between Treg absolute numbers and immune activation [88,106,116].

- Under an effective HAART, a high percentage of Treg does not seem to be an advantage: 1) their percentages decrease with effective HAART; 2) a high percentage of Treg seems to limit immune reconstitution in patients under HAART [117,126-128]; 3) and also, in chronically SIV-infected HAART-treated macaques, the administration of anti-CTLA-4 blocking antibody (to block Treg function) was found to have beneficial virological effects [129]; 4) moreover, in two large global randomized controlled clinical trials (ESPRIT and SILCAAT), although supplementary IL-2 treatment resulted in a substantial and long-standing increase in

$CD4^+$ T cell counts compared to antiretroviral therapy alone [130], it has been demonstrated that a substantial proportion of the $CD4^+$ T cells expanded shared phenotypic, functional and molecular characteristics with Treg. Apparently, this seems to be the cause of the unexpected clinical outcome observed in those patients - they presented more potentially life-threatening events [36].

While it is well known that HAART leads to $CD4^+$ T cell counts recovery preventing AIDS related complications, it does not prevent several complications typically associated with aging which tend to prematurely commit long-term treated patients [131,132]. These conditions seem to be related in part to immune activation [131,132]. Under successfully treatment, $CD4^+$ and $CD8^+$ T-cell activation dramatically decrease with viral suppression, but remains higher compared to healthy controls [131]. Treg frequency seems not to be crucial in reducing immune activation, as in most studies a successful treatment results in decrease of both immune activation and Treg frequency [88,89,101,102,131]. However, in the viral control situation under HAART, Treg frequency was found to negatively correlate with residual immune activation [133,134]. Interestingly, this negative relationship between Treg frequency and $CD8^+$ T-cell activation was lost after treatment interruption (that resulted in viral rebound) [124]. Also, in HIV elite controllers, peripheral T-cell activation is lower than in patients with progressive disease, but higher than in HAART-treated aviremic patients, an observation that could be related to lower Treg frequency in HIV controllers than in successfully HAART-treated patients [103]. We can then conclude that in most HIV controllers, low levels of Treg could contribute to the high specific anti-HIV $CD8^+$ T-cell responses described, but also for the relatively higher immune activation levels compared to HAART-treated patients [91].

Hence, it appears that Treg may be able to control low levels of T-cell activation, but the effect may not be adequate to control high levels of immune activation as is often present, especially when levels of viral replication are high [91]. This could happen due to a decrease in the absolute number of Treg or because Treg, due to their plasticity in some environments rich in pro-inflammatory cytokines and Treg-attracting chemokines, can enter inflammatory locations, but after an initial anti-inflammatory effort could change function, be induced to secrete the pro-inflammatory cytokine IL-17 and contribute to tissue damage and immune activation [39,48,91,135].

To conclude peripheral and mucosal Treg seem to trigger several effects that could be either beneficial or detrimental during the HIV infection, each of them playing different roles depending on the phase of the disease and possible other characteristics of individuals patients..

Treg may have an overall beneficial effect during early acute HIV infection; before HIV-specific immune responses are fully activated, by controlling T cell activation and decreasing the availability of target cells for HIV replication and transmission [114,115,118]. In contrast, during the late acute and chronic phases, increased Treg frequency may have an overall-negative role, as the Treg suppressive effect on HIV antiviral immune responses predominates and those cells seem be unable to counter immune activation [87,88,90,113,117,124]. In addition, a hampered $CD4^+$ T cell reconstitution likely due to exacerbated fibrosis of lymphoid tissues could contribute to this overall negative role [85]. The inhibition of the lymphopenia-induced proliferation, by these cells, could also explain the poor immune reconstitution sometimes observed [54,117,127]. However in virological suppressed patients under HAART, a higher Treg frequency could account for a reduced immune activation [124].

The influence of Treg in HIV infection/AIDS outcome seems to depend on the equilibrium between the negative effect of suppressing desired effector T cell responses and the positive effect of decreasing unwanted/exacerbated immune activation. If we could identify a particular subset of Treg able to suppress immune activation without suppress pathogen-specific immune response and unable to reprogrammed themselves, it would be of great interest for immune-based therapy in chronic infectious diseases namely the HIV infection. The boundary between the beneficial effect and adverse effect of Treg seems to be very weak, and any impetuous action may cause the disruption of this equilibrium and have disastrous consequences.

Several strategies have been developed aiming towards the abrogation of Treg that could be helpful in some diseases. The use of those strategies could be useful in HIV infection if the real role of Treg in the different phases of disease and/or the patient variables that influence this outcome would clearly known. To achieve this goal, much research has to be performed.

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Chapter 2

Results

2.1. Construction of a Clinical and Biologic Database

2.1.1. Introduction

The emergence of HAART to treat HIV-infected patients markedly reduced the morbidity and mortality associated with this infection. This is mainly achieved due to immune system recovery. However, some of the patients, although being under effective HAART, do not reach a normal (700-1100 cells/mm^3), a near normal (>500 cells/mm^3) or, at least, a satisfactory $CD4^+$ T cell counts (a number higher than a threshold of 250-350 cells/mm^3, a level that would enable patients to be protected from more severe opportunistic infections). For this reason, these individuals are more prone to suffer from AIDS-related and AIDS non-related complications and death. There is considerable controversy regarding the relative contribution of various mechanisms of sub-optimal T cell recovery.

Trying to better understand the role of various interveners during the immune reconstitution, we gathered a set of clinical, imagiological, laboratorial, and biological data from HIV-infected patients undergoing HAART.

The patients were selected from the hospital where these patients undergo medical care (HJUU/CHP). Blood samples of these patients were collected at HJUU/CHP and processed at the ICVS/ECS/UM. At the ICVS, the blood samples were analyzed, plasma was isolated and frozen and surplus blood cells were frozen for future analysis. Computer databases for all the data (clinical, imagiological and laboratorial data) were generated.

2.1.2. Construction of a Cross-Sectional and a Longitudinal Cohort to Study Immune Reconstitution among HIV-Infected Patients under HAART

Patients were enrolled for two independent type of studies: cross-sectional and longitudinal studies:

2.1.2.1. The Cross-Sectional Study

The cross-sectional study allowed to promptly examine a segment of patients infected by HIV and on HAART, and evaluated several parameters of their immune reconstitution in a single time-point taking into account information on their clinical history.

The number of patients included in this cohort was not pre-defined, and until now, 123 adult patients were included, with the following criteria: 1) infection by HIV-1; 2) receiving or not HAART (regardless of the scheme used); 3) when on HAART, presumably being regular in therapy compliance. All participants have signed an informed consent (Annex 1) on a volunteer basis after explanation and prior to their enrollment in the study. This study was approved by the institutional review board of the HJUU/CHP under the protocol number 168/CES [addenda number 127/12 (NA-DEFI/089-CES)] (Annex 2 and 3).

Participants in this study were randomly chosen among patients that were attending the hospital for routine analysis, on a day also randomly chosen, and subjected in that day to the only visit of the study. The patients' physician informed and explained to patients about the study and if patients agreed in participating, a written informed consent was sign. Apart from the blood collected for the pre-programmed follow-up evaluation of these patients (performed at the hospital's reference laboratory), additional blood samples were collected into heparinized tubes and transported to the ICVS to be processed on the same day as described in section 2.1.3.

Clinical and demographic data was collected by patients' physician from the clinical file (using a specific form – Annex 4) such as: patient's epidemiological characteristics (gender and age); transmission mode of HIV; probable date of infection; date of HIV infection diagnosis; clinical history including infections/tumors HIV/AIDS-related and other relevant clinical conditions; hepatitis C virus (HCV) or B virus (HBV) co-infections. For patients under HAART: HAART onset date, nadir and/or baseline value of $CD4^+$ T cell counts, clinical and laboratorial progression since HAART was initiated, and HAART schemes history and respective compliance. Viral load and $CD4^+$ T cell counts were determined by referenced laboratory of the hospital on the same day of the visit.

A computer database was constructed and data analysis performed. This database allowed the study described in Subchapter 2.2 and other studies that resulted in Master's thesis and which are currently supporting other studies.

Epidemiological, virological and immunological characteristics of the cohort (n=123) are described in Table 1.

Paramater	Results
Age, in years Mean ± SD (range) Median, (IQR) N=123	45,19 ± 9,10 (24-76) 44,00 (51–38)
Sex – male, female n (%)	103 (83,7), 20 (16,3)
Patients under HAART Yes (%)/No (%) N=123	104 (84,55)/19 (15,45)
Time between HAART onset and study visit, in years Mean ± SD (range) Median, (IQR) N=102	6,38 ± 4,57 (0–17) 5,50 (11–2)
CDC CD4 cell category [2] n (%) N=119	(1) ≥500/mm^3 – 56 (47,06) (2) 200-499/mm^3 – 54 (45,38) (3) <200/mm^3 – 9 (7,56)
CD4$^+$ T cell count (cell/mm^3) Mean ± SD (range) Median, (IQR) N=119	537,70 ± 282,24 (41–1372) 486,00 (747-345)
CD4$^+$ T cell count (cell/mm^3) increase (CD4$^+$ T cell count: Study visit - HAART onset) Mean ± SD (range) Median, (IQR) N=101	309,88 ± 224,28 (-161 - +909) 283,00 (432,00-132,50)
Viral load (VL) copies/ml On treatment Mean ± SD (range) Median, (IQR) N=99/104	<50 (virological supressed): 81 (81,82%) ≥50: 18 (18,18%): 33510,28 ± 101351,98 (51-413000) 296,50 (2057,50–93,50)
Viral load (VL) copies/ml Without treatment Mean ± SD (range) Median, (IQR) N=17/19	96335,29 ± 178610,44 (1670-639000) 25800,00 (100400,00–4785,00)

Tabela 1. Epidemiological, clinical, virological and immunological characteristics of the cohort.

2.1.2.2. The Longitudinal Study

The longitudinal study aims to better understand the temporal order of events to determine the direction and the magnitude of potential cause-and-effect relationships between factors/interveners and outcomes (several grades of immune reconstitution process after HAART onset). Patients were enrolled according to pre-defined parameters and followed-up in defined time-points according to a protocol described below. In each time-point, coincident with routine appointments, patients were questioned about symptoms, underwent a medical examination and blood was collected and analyzed at the hospital (routine analysis) and at the ICVS. The data was collected and organized in the database. The first study (described in subchapter 2.3) describes

the evolution of Treg along therapy, an obvious follow up of our first report using the cross-sectional study. This longitudinal cohort is presently being used for other studies.

In the longitudinal study, a number of 100 patients were pre-defined to be included (a number of patients that it will be feasible to clinical care and whose blood will be economically feasible to be analyzed), and so, 100 adult patients were enrolled with the following criteria: 1) infected by HIV-1; 2) naïve on HAART; 3) having criteria to initiate HAART. All participants have signed an informed consent (Annex 5) on a volunteer basis after explanation of the entire protocol. A local Ethical Committee approval was received for the study (reference 168/CES on 2th October 2009) (Annex 6).

The patients were included between 29-04-2010 and 11-10-2012 (35 in 2010, 50 in 2011 and 15 patients in 2012) when our aimed number of patients - 100 - was achieved. The start of HAART defined the study visit "0" or baseline visit. The decision to initiate HAART was individualized and upheld by the existence of at least one of the following elements: symptomatic clinical manifestations (presence of previously designed as category B and of any of the AIDS-defining conditions by the Centers for Disease Control and Prevention – CDC [2,3]); number of $CD4^+$ T lymphocytes or $CD4^+$ T lymphocytes percentage of total of lymphocytes, <350 cells/mm^3 or <14%, respectively; plasma viral load value >100000 copies/ml in at least two determinations; presence of co-morbidities (cardiovascular disease or high risk for cardiovascular disease, HIV associated nephropathy – HIVAN, chronic hepatitis B virus or hepatitis C virus infection) or age >60 years. The patient's level of willingness and readiness was assessed, indorsed, and confirmed before HAART initiation [4-7].

Antiretroviral Therapy

The HAART scheme chosen for each patient took into consideration scientific policy and National and International Guidelines and the price [4-7]. The first option was a combination of two Nucleoside Reverse Transcriptase Inhibitors (NRTIs) and one Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTI), of which efavirenz (EFV) was the first option. The choice of NRTIs depended on the viral load; if the viral load value was below 100000 copies/ml, with no cardiovascular risk, and the allele of major histocompatibility complex was not HLA-B*5701, the option was abacavir (ABC) and lamivudine (3TC) coformulation (Kivexa®), if the viral load was above 100000 copies/ml, the option was then tenofovir (TDF) and emtricitabine (FTC) coformulation (Truvada®). When NNRTIs - EFV - could not be used (existence of hipersensibility,

mutations for resistance, or co-morbidities like psychiatric diseases that preclude his use), a Protease Inhibitor (IP) boosted with ritonavir was chosen being darunavir once daily, the first option. One patient, a woman, initiated zidovudine (AZT) + 3TC + nevirapine (NVP); she was suffering from cryptococcal meningitis and CMV encephalitis so the avoidance of EFV was due to its potential neuropsychiatric effects that could worsen the patient's clinical state and, also, the use of potentially better central nervous system (CNS)-penetrating drugs like AZT or NVP (her viral load in cerebrospinal fluid - CSF - was about 157000 copies/ml) was tried. Two other patients initiated, as the third drug, lopinavir boosted with ritonavir (LPV/r); they were the first two patients using IPs in this cohort, they had CNS infections (tuberculosis meningitis and CMV encephalitis) and a high CSF HIV viral load, and LPV/r at the time was thought to be one of the more CNS-penetrating drugs.

Protocol of study visits

The patients were evaluated prior to initiation of HAART, the day they started HAART (baseline visit) and regularly thereafter following a protocol (Annex 7). The duration of this study was initially planned to be of three years. However, to have more time of follow-up would be suitable to the purposes of our aim; to better characterize the direction and the magnitude of cause-and-effect relationships between variables, it was decided to keep the patients in semiannual study visits after completion of three years until, at least, five years. At present, the study is yet ongoing.

Prior to enrolment, the study and the entire protocol were explained to patients that signed the informed consent. In some cases, this visit was the first at the hospital and the first medical visit after the patient was informed that he/she was infected with HIV. Therefore, before the study explanation, a long conversation was taken, explaining to the patient the natural course of the infection, what are the medical tools to counteract the disease's evolution, all the scientific advances made since the beginning of the epidemic, the therapy schemes and their potential adverse effects, and the need to be adherent to the therapies due to the ability of HIV to acquire mutations conferring resistance to HAART. Additionally, recommendations as how to lead a healthy life and about precautions to take to avoid the infection of others were provided. Also in this visit, a complete and exhaustive clinical assessment (questions about medical history, history of present illness if it was the case, and physical examination) was performed.

During the baseline visit, HAART was given according to a resistance test and to other conditions already described. Again an explanation regarding the medication was also given. In this visit, the first blood collection to be analyzed at ICVS was performed.

In the follow-up visits, an evaluation consisting of clinical, analytical and imaging studies was performed. The analytical study included study routine, assessment of $CD4^+$ T lymphocytes and viral load and other analyses to evaluate potential infections (latent or active). All blood collections were performed at the hospital laboratory on the same day of the medical visits. On each visit, additional blood was also collected and transported to the ICVS, where it was processed the same day.

The study visits were always made by the physician responsible for the patient/study – Ana Horta. Additional visits, blood collects, or hospitalizations were performed when the clinical condition of the patient demanded it. These decisions were made also by Ana Horta and when she was not available, made by the emergency physician. When hospitalized, patients were assigned to a physician in the rotation scheme instituted by the Infectious Diseases Department.

Epidemiological, clinical, virological and immunological characteristics of this cohort at baseline are described in Table 2. Primary resistance to HAART (to NRTIs, to NNRTIs, and/or IPs) was present in 25 patients (some of them revealing mutations conferring resistance to more than one class of antiretroviral drugs) and the resistance mutations involved are reported in Table 3. In Table 4, indicator conditions defining AIDS [3]) present at baseline are shown (in six cases, more than one condition were simultaneously present).

When an AIDS-indicator condition was diagnosed previous to HAART initiation, the median period of time between the diagnosis/treatment onset for that condition, and the beginning of HAART (baseline) was 69,35 ± 75,19 day, 12 – 360 (Mean ± SD, range) or 44,50, 90,00 - 19,50 (Median, IQR).

Symptomatic conditions that are not included in AIDS-indicator condition definition, but are attributed to a cell-mediated immunity defect or for which the clinical course or management is complicated by HIV infection (prior classified in the classification from 1993 by CDC, as category B symptomatic-indicator conditions) [2] presented at baseline or referred as recently presented by patients are shown in Table 5. In sixteen patients, more than one condition was present. Others medical conditions present at baseline and not directly related to HIV infection are reported in Table 6. Data related to infection by hepatitis virus, at baseline, are shown in

Table 7. Baseline HAART combinations prescribed for the 100 patients at baseline are shown in Table 8.

Paramater	Results
Age, in years, N=100 Mean, SD, (range) Median (IQR)	40,44 ± 10,42 (22–67) 41,00 (46,75-32,00)
Sex - male, female - n (%), N=100	79 (79), 21 (21)
Transmission mode of HIV n=% N=100	IVDUs – 20 Heterosexuals – 43 IVDUs and Heterosexuals – 5 MSM – 29 Unknown – 3
HIV subtype and circulating recombinant forms (CRFs) n=% N=100	B – 47, G – 25, C – 10 Others – 9 (A-3, A/D-2, CRF02_AG-1, CRF14_BG-1, F-2) Unknown – 9
Time between diagnosis of HIV infection and baseline, in months, N=100 Mean, SD, (range) Median (IQR)	25,64 ± 34,97 (0-178) 8,00 (37,75-2,25)
Time between primo infection by HIV (known or likely) and baseline in months, N=17 Mean, SD, (range) Median (IQR)	28,59 ± 24,93 (3-76) 26,00 (50,00-5,00)
CDC clinical category [2] n=%, N=100	A – 30, B – 44, C – 26
CDC CD4 cell category [2] n=% N=100	(1) ≥500/mm^3 – 7 (2) 200-499/mm^3 – 49 (3) <200/mm^3 – 44
CDC stage C or (3) (AIDS) [2,3] n=%, N=100	48
CD4$^+$ T cell count (cell/mm^3), N=100 Mean, SD, (range) Median (IQR)	244,57 ± 192,37 (4-1033) 247,50 (320,50-92,00)
CD8$^+$ T cell count (cell/mm^3), N=99 Mean, SD, (range) Median (IQR)	1025,19 ± 570,48 (150-2776) 897,00 (1353,00-605,00)
Viral load - copies/ml, log 10 copies/ml Mean, SD (range) Median (IQR) N=100	859025,90 ± 1844752,70 (6790-10M), 5,42 ± 0,64 (3,83–7,00) 228500,00 (89400-583000), 5,36 (4,95–5,77)
Presence of mutations associated with resistance to drugs, by genotypic assays, N=96	Yes - 25 (26,0%) No – 71 (74,0%)
HAART resistance mutations detected by genotypic assays (detected in 25 patients)	NRTI – 3 (12,0%) NNRTI – 10 (40,0%) IP – 15 (60,0%) [NNRTI + IP – 3 (12,0%)]
HLA-B*5701 N = 76	Positive - 3 (4%) Negative – 73 (96%)

Table 2. Epidemiological, clinical, virological and immunological characteristics of the cohort at baseline. SD – standard deviation, IQR – interquartile range, IVDUs – intravenous drug users, MSM – men having sex with others men, CDC – Centers for Disease Control and Prevention, HAART – antiretroviral therapy, NRTIs - Nucleoside Reverse Transcriptase Inhibitors, NNRTIs - Non-Nucleoside Reverse Transcriptase Inhibitors, IPs - Protease Inhibitors, HLA-B*5701 – allele of major histocompatibility complex, class I, B.

NRTIs resistance mutations (3 patients)	T69N	2
	T215S	1
	Total	**3**
NNRTIs resistance mutations (10 patients)	K101E	1
	K103N	2
	E138A	1
	V179D	3
	Y188L	1
	G190A	1
	G190E	1
	Total	**10**
IPs resistance mutations (15 patients)	L10I	2
	K20I	7
	M46L	1
	T74S	4
	L90M	6
	Total	**20**

Table 3. Resistance mutations to HAART presented at baseline (in 25 patients of 96 tested). NRTIs - Nucleoside Reverse Transcriptase Inhibitors, NNRTIs - Non-Nucleoside Reverse Transcriptase Inhibitors, IPs - Protease Inhibitors.

AIDS-indicator conditions		**N (26 patients)**	
Mycobacterium tuberculosis disease	Pulmonary	11	7
	Extrapulmonary		4
Pneumocystis jiroveci pneumonia		10	
Candidiasis esophageal		4	
Kaposi's sarcoma		4	
Cytomegalovirus (CMV) encephalitis, esophagitis and/or colitis		3	
Mycobacterium avium complex disease		1	
Cryptococcal meningitis		1	
Cerebral Toxoplasmosis		1	

Table 4. Indicator conditions in case definition of AIDS at baseline (in 26 of 100 patients) (in six cases, more than one condition were simultaneously present).

ategory B symptomatic conditions	**N (44 patients)**
Oropharyngeal candidiasis (thrush)	38
Herpes zoster (shingles), involving two or more episodes or at least one dermatome	9
Diarrhea lasting >1 month	4
Cervical dysplasia (moderate or severe)/cervical carcinoma in situ	1
Vulvovaginal candidiasis, persistent or resistant	1
Angular cheilitis	1

Table 5. Category B symptomatic conditions (by CDC) [2] present or referred as recently presented by 44 of 100 patients at baseline (sixteen patients presented more than one condition).

Medical conditions not related to HIV infection	N (53 patients)
Syphilis	VDRL positive – 17 FTA-ABS positive - 28 (Latent – 15, Secondary – 2, Early disease - 2 Cured - 9)
Genital Herpes	6
Condyloma acuminata	5
Molluscum contagiosum	2
Neisseria gonorrhoeae urethritis	1
Community-acquired Pneumonia	6
DPOC	3
Urinary tract infection	3
Psoriasis	3
Neoplasms	3
Diabetes Mellitus	3
Gallstones	2
Nephrolithiasis	1
Porphyria Cutanea Tarda	1
Acute myocardial infarction	1
Situs inversus	1
Influenza A/H1N1	1
Gastroenteritis	1

Table 6. Others medical conditions present at baseline and not directly related to HIV infection (in 53 of 100 patients). VDRL – Venereal Disease Research Laboratory test, FTA-ABS – Fluorescent Treponemal Antibody Absorption

Paramater	Results
Anti-HCV positive N = 100	30 (24 – HCV RNA +; 6 patients – spontaneous clearance)
HCV genotypes (performed in the 24 patients with HCV RNA+)	G1 – 15 (68,2%): 1a - 12, 1b – 2, 1 - 1 G3 – 5 (22,7%) G4 – 2 (9,1%) G? - 2
HCV RNA (IU/ml) Median, SD, (range) (performed in the 24 patients with HCV RNA+)	5170180,83 ± 4861594,17 (609 - 15400000)
HBsAg positive (N = 100) HBV DNA (IU/ml)	1 (Baseline HBV DNA = 171000000)

Table 7. Data related to infection by hepatitis virus, at baseline. Anti-HCV – Hepatitis C virus antibody, HBsAg – Hepatitis B surface antigen.

NRTIs pairs		N (100 patients)	Total	
FTC + TDF		78	100	
3TC + ABC		21		
AZT + 3TC		1		
3th Drug		N (100 patients)	Total	
NNRTI	EFV	85	86	100
	NVP	1		
IP	DRV/r	12	14	
	LPV/r	2		

Table 8. Initial regimen of HAART prescribed to the 100 patients, at baseline.
NRTIs - Nucleoside Reverse Transcriptase Inhibitors, NNRTIs - Non-Nucleoside Reverse Transcriptase Inhibitors, IPs - Protease Inhibitors, FTC – Emtricitabine, TDF – Tenofovir, 3TC – Lamivudine, ABC – Abacavir, EFV – Efavirenz, NVP – Nevirapine, DRV – Darunavir, r – Ritonavir as boosted, LPV/r – Lopinavir boosted with ritonavir

To evaluate potential latent or active tuberculosis, a tuberculin skin test (TST or Mantoux) and interferon-gamma release assays (IGRAs) - Quantiferon® were performed in the vast majority of the patients. The Mantoux test was performed using the intradermal injection of five tuberculin units (5TU) of purified protein derivative (PPD). The results obtained are shown in Table 9.

Diagnostic test	Results
Tuberculin skin test (TST) N=97	Negative – 86 Positive (> 5mm) – 11 (Tuberculosis disease - 2 Latent Tuberculosis assumed - 9)
IFN-Gamma release assay (IGRA) Quantiferon® N = 98	Negative – 84 Positive – 8 (Tuberculosis disease – 1 Latent Tuberculosis assumed - 7)

Table 9. Tuberculin skin test and Interferon-gamma release assay results at baseline.

Thymus computed tomography

Since the thymus has been shown to play an important role on the immune reconstitution of HIV-infected patients, a mediastinic non-contrast computed tomography (CT) was performed to evaluate thymic structure and dimension in 46 patients on the baseline visit (or just before) and upon 1 year on HAART, at Computerized Medical Service Image, SA (SMIC), Porto. The determination of the thymic volume and index were performed independently, and in a blinded manner by two appraisers. The results at baseline and at 1 year of follow-up were thereafter compared, between each other and to flow cytometry results. Those results were presented in an integrated Master thesis in Medicine (unpublished data) and are presently been processed for a future publication.

A database of healthy controls (age and gender matched) was constructed from individuals recruited from both institutions. The establishment of this database will not be described in the context of this work. The data base of controls included 252 adults without HIV infection.

2.1.3. Samples Processing and Data Analysis – Work Performed at Laboratory

The processing of samples from patients and healthy controls, regardless the type of study or the time-point of the longitudinal study was essentially the same. Whenever there was a difference, this was highlighted. Importantly, all the blood was sent to the ICVS and processed on the same day it was collected.

The blood samples were processed as depicted in Figure 1. A blood sample collection into a tube without anticoagulant (performed only for longitudinal study) was centrifuged (2500 rpm, 10 min), the serum was collected and stored in aliquots at -80°C for subsequent analysis of different molecules such as cytokines and/or chemoquines.

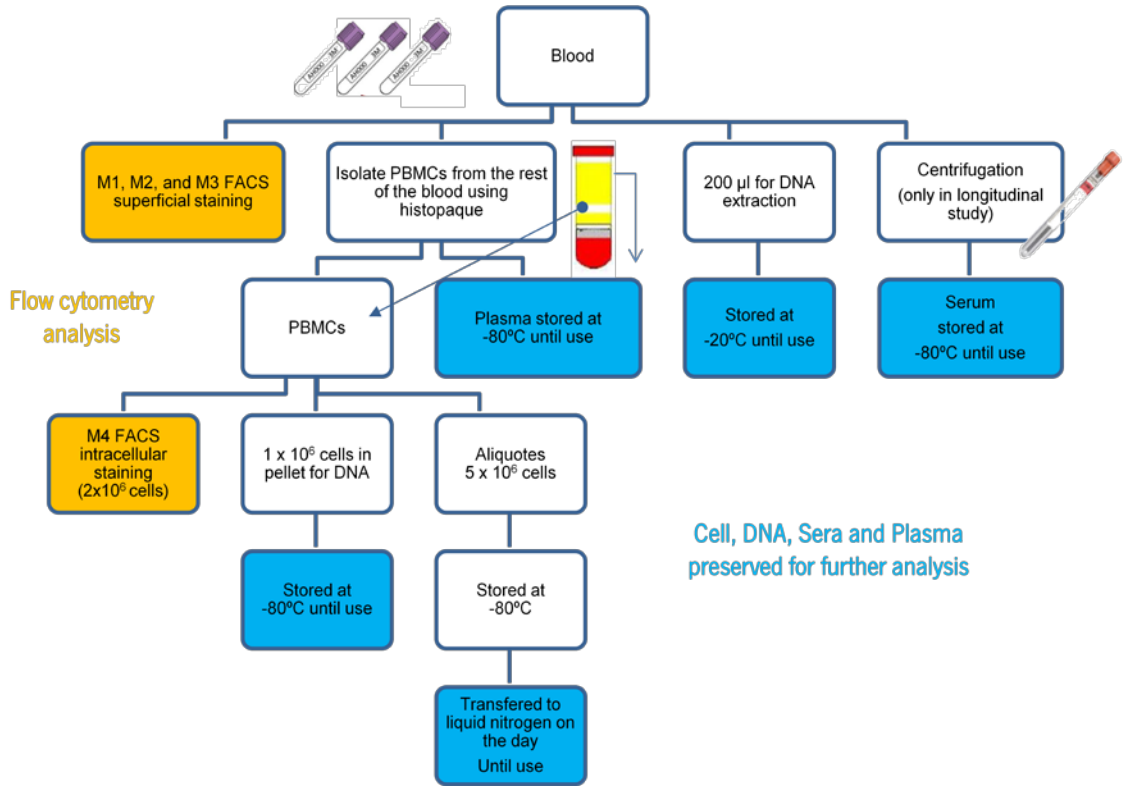

Figure 1. Several tasks performed at ICVS with blood transported from UHU/CHP. Boxes in yellow: blood processed for multiparametric flow cytometry analysis after staining with a combination of monoclonal antigens – analysed presently. Boxes in blue: to be processed and analyzed in the future FACS – Fluorescence-activated cell sorting, PBMCs - Peripheral Blood Mononuclear Cells

A proportion of blood was processed for multiparametric flow cytometry analysis to allow the phenotypic characterization of distinct cell populations. Four different sets of monoclonal antibodies combinations were used (Table 10); three of them performed on total blood and uniquely for the identification of surface markers (M1, M2, and M3) and the fourth performed on peripheral blood mononuclear cells (PBMCs) to evaluate surface and intracellular markers (M4). A brief description of the protocol performed is described afterwards.

Staining	Antibody	Clone	Flurofore	Aim
M1	CD3	OKT3	PE	Granulocytes B and T cells
	CD15	W6D3	PerCP	
	CD19	HIB19	FITC	
	TCRαβ	IP26	Alexa Fluor 647	
M2	CD3	OKT3	Pacific Blue	$CD4^+$ and $CD8^+$ T cells activation
	CD4	RPA-T4	APC-Cy7	
	CD8	RPA-T8	APC	
	CD45RA	HI100	FITC	
	CD45RO	UCHL1	PerCP-Cy5,5	
	CD69	FN50	PE	
	HLA-DR	L243	PE-Cy7	
M3	CD3	OKT3	PE	$CD4^+$ and $CD8^+$ T cells subsets: Recent thymus emigrant (RTE) Naive Memory Central memory Effector memory
	CD4	RPA-T4	APC-Cy7	
	CD8	RPA-T8	BV	
	CD31	WM59	PE-Cy7	
	CD45RA	HI100	FITC	
	CD45RO	UCHL1	PerCP-Cy5,5	
	CCR7	TG8/CCR7	Alexa Fluor 647	
M4	CD3	UCHT1	V500	Treg and its subsets: Naives Memory RTE Proliferation
	CD4	RPA-T4	APC-Cy7	
	CD25	BC96	APC	
	CD31	WM59	PE-Cy7	
	CD45RA	HI100	Pacific Blue	
	CD127	AO19D5	PerCP-Cy5,5	
	FOXP3	PCH101	PE	
	Ki67	MOPC-21	FITC	

Table 10. Combination of monoclonal antibodies performed. Each antibody was titrated using serial dilutions until optimal concentrations was determined. All antibodies were from Biolegend except for the anti-FOXP3 (eBiosciences), anti-CD3 V500 (BS Horizon) and anti-Ki67 (BD Biosciences).

Isolation of PBMCs

PBMCs were isolated using gradient centrifugation with Histopaque 1077 (Sigma-Aldrich) (Figure 1). After centrifugation (1500 rpm, 30 min), at room temperature, the plasma (upper phase) was collected to cryovials (stored at -80°C) and the interface between the plasma and the Histopaque 1077, containing PBMCs, was collected to a new tube with apyrogenic phosphate buffered saline (aPBS) (GIBCO/Life Technologies). After washing twice with the aPBS, the cell pellet was re-suspended in 500 μL of RPMI supplemented with 10% fetal bovine serum (FBS) (both from GIBCO/Life Technologies) and cells were counted using trypan blue to exclude dead cells. After taking cells to perform fluorescence-activated cell sorting (FACS) staining (M4), the remaining cells were frozen in aliquots of $5x10^6$ PBMCs each, in a media containing 80% RPMI, 10% FBS and 10% DMSO.

Staining for surface markers for flow cytometry

For the surface marking, 100 (M1 and M2) or 200 μl (M3) of whole blood were incubated for 15 min, at room temperature, with a defined set of monoclonal antibodies (Table 10). Afterwards, erythrocytes were lysed by incubation with FACS Lysis Buffer (BD Biosciences, San Jose, CA, USA) for 15 min at room temperature. Cells were washed with FACS buffer.

Staining for intracellular markers for flow cytometry

By the time these studies were initiated, no reliable methods were available for the staining of FOXP3 on total blood cells. For that reason, this staining had to be performed in isolated PBMCs.

From the PBMCs suspension previously prepared, two million of fresh PBMCs were stained for M4 (Table 10). Cells were first incubated with the antibodies for the surface markers for 15 min at room temperature. After the excess antibody was washed out, cells were fixed and permeabilized using the FOXP3 Staining Buffer Set (eBioscience, San Diego, CA, USA). Cells were incubated with a mix of anti-Ki67 and anti-FOXP3 (intracellular markers) and after being washed with permeabilization buffer and FACS buffer.

Sample acquisition on the flow cytometer

All samples were acquired the same day they were processed on a BD LSR II flow cytometer (equipped with 3 lasers and 8 fluorescence detectors) using FACS DIVA software

(Becton and Dickinson, NJ, USA). Data was analysed using FlowJo Software (Tree Star, OR, USA).

2.1.4. Bibliography

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2.2. Poor Immune Reconstitution in HIV-Infected Patients Associates with High Percentage of Regulatory CD4+ T Cells

Ana Horta[1,2,3]*, Claudia Nobrega[1,2]*, Pedro Amorim-Machado[1,2], Vitor Coutinho-Teixeira[1,2], Palmira Barreira-Silva[1,2], Susana Boavida[3], Patrício Costa[1,2], Rui Sarmento-Castro[1,2,3], António Gil Castro[1,2], Margarida Correia-Neves[1,2]

1 Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal.

2 ICVS/3B's, PT Government Associate Laboratory, Braga/Guimarães, Portugal.

3 Infectious Diseases Service of the Centro Hospitalar do Porto, Hospital Joaquim Urbano Unit, Porto, Portugal.

* These authors contributed equally to this paper

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Poor Immune Reconstitution in HIV-Infected Patients Associates with High Percentage of Regulatory CD4$^+$ T Cells

Ana Horta[1,2,3☯], Claudia Nobrega[1,2☯], Pedro Amorim-Machado[1,2], Vitor Coutinho-Teixeira[1,2], Palmira Barreira-Silva[1,2], Susana Boavida[3], Patrício Costa[1,2], Rui Sarmento-Castro[1,2,3], António Gil Castro[1,2], Margarida Correia-Neves[1,2]*

1 Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal, **2** ICVS/3B's, PT Government Associate Laboratory, Braga/Guimarães, Portugal, **3** Infectious Diseases Service of the Centro Hospitalar do Porto, Hospital Joaquim Urbano Unit, Porto, Portugal

Abstract

CD4$^+$ regulatory T cells (Tregs) are essential for the maintenance of the immune system's equilibrium, by dampening the activation of potential auto-reactive T cells and avoiding excessive immune activation. To correctly perform their function, Tregs must be maintained at the right proportion with respect to effector T cells. Since this equilibrium is frequently disrupted in individuals infected with the human immunodeficiency virus (HIV), we hypothesize that its deregulation could hamper immune reconstitution in patients with poor CD4$^+$ T cell recovery under highly active antiretroviral therapy (HAART). We analysed Tregs percentages amongst CD4$^+$ T cells in 53 HIV-infected patients under HAART, with suppression of viral replication and distinct levels of immune reconstitution. As controls, 51 healthy individuals were also analysed. We observed that amongst the patients with Nadir values (the lowest CD4$^+$ T cell counts achieved) $<$200 cells/μL, the individuals with high Tregs percentages ($\geq$10% of total CD4$^+$ T cells) had the worse CD4$^+$ T cell reconstitution. In accordance, the well-described direct correlation between the Nadir value and CD4$^+$ T cell reconstitution is clearly more evident in individuals with high Tregs proportions. Furthermore, we observed a strong negative correlation between Tregs percentages and CD4$^+$ T cell recovery among immunological non-responder HIV$^+$ individuals. All together, this work shows that high Tregs frequency is an important factor associated with sub-optimal CD4$^+$ T cell recovery. This is particularly relevant for immunological non-responders with low Nadir values. Our results suggest that the Tregs proportion might be of clinical relevance to define cut-offs for HAART initiation.

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* E-mail: mcorreianeves@ecsaude.uminho.pt

☯ These authors contributed equally to this work.

Introduction

Infection with HIV initiates a series of events that ultimately lead to profound immunosuppression, caused by functional abnormalities in the immune system, mainly due to severe depletion of CD4$^+$ T cells [1].

The introduction of HAART has led to very important declines in both mortality and morbidity due to HIV infection [2]; however, even though many patients steadily recover their CD4$^+$ T cell compartment over several years post-HAART initiation, the degree of immune recovery achieved is highly variable. On this, studies indicate that even after several years of treatment, a proportion of patients (from 15% to 40%) feature abnormally low CD4$^+$ T cell counts despite suppression of HIV replication [3,4,5,6]. This group of individuals is referred to as immunological discordants or non-responders and, unlike full responders, they are at increased risk of clinical progression to acquired immunodeficiency syndrome (AIDS)-related and non-related illnesses and death [2].

Sub-optimal CD4$^+$ T cell recovery may result from excessive/premature cell death, decreased peripheral proliferation and/or reduced production of these cells by the thymus. Several factors have been suggested to contribute to this limited ability of the CD4$^+$ T cell compartment to normalise (reviewed in [7]) such as advanced age [8], low baseline CD4$^+$ T cell counts [6,8,9], residual HIV replication [10], chronic immune activation [11], abrogated thymic function [12,13], gender [14,15] and genetic polymorphisms associated with increased programmed cell death [16,17]. While all these factors are definitely relevant in establishing different immune reconstitution profiles, there may be other factors also contributing to this process [7].

Tregs are essential for the maintenance of self-tolerance and immune homeostasis [18] and have been widely studied in the context of HIV infection. Most studies have focused on whether or not these cells are directly infected by HIV, to what extent are they

depleted/expanded, and their role during the course of disease progression from HIV infection to AIDS. The ability of HIV to directly infect Tregs is still a subject of debate. Whilst it has been reported that they are susceptible to HIV infection *in vitro* [19,20], other studies showed that exposure of Tregs to HIV selectively promotes their survival via a CD4-gp120–dependent pathway [21]. Moreover, the accumulation of Tregs in the gut or in the tonsils of HAART-naïve HIV^+ patients also argues against increased killing of these cells in compartments where viral replication is occurring [22,23].

During the course of disease progression in HAART-naïve patients, Tregs seems to act as a double-edged sword. On one hand frequencies of these cells have been shown to negatively correlate with the levels of immune activation [24,25,26,27,28], which is one of the key contributors to HIV disease progression [29,30,31]; on the other hand, high levels of Tregs have also been linked with suppression of HIV-specific $CD4^+$ and $CD8^+$ T cell activity and, thus, in this way Tregs could be linked to a worse disease prognosis [32,33,34]. Tregs have also been investigated in the context of HAART-associated immune reconstitution, although to a much lesser extent. Results so far also lack a consensus. Some authors found elevated levels of Tregs in aviremic HIV^+ patients in comparison to healthy controls [35,36,37,38], whilst others described Tregs frequencies as following a "biphasic curve" during the first year of HAART [39], with an initial increase and a subsequent return to levels comparable to controls [22,23,39]. The observation that the proportion of Tregs in HAART-treated aviremic HIV^+ individuals is quite diverse prompted us to explore whether distinct values were associated with differences in $CD4^+$ T cell recovery. Thus, we investigated how the percentage of Tregs within $CD4^+$ T cells correlated with $CD4^+$ T cell count recovery and other parameters of immune reconstitution in HAART-treated HIV^+ individuals. Understanding the mechanisms that limit T cell recovery during HAART is essential to help adjust guidelines for HAART initiation, and to prompt investigation of complementary immune therapies that could enhance immune reconstitution.

Materials and Methods

Ethics Statement

This study was approved by the institutional review board of the Hospital Joaquim Urbano under the protocol number 168/CES [addenda number 127/12 (NA-DEFI/089-CES)]. Study subjects gave written, informed consent prior to their participation.

Study population

A cross-sectional study was performed with 53 HIV^+ individuals recruited from Hospital Joaquim Urbano, Porto, Portugal (43 ± 8 years old, range 31 to 58 years old; 79% were males) and 51 healthy individuals recruited from the same hospital and from the Life and Health Sciences Research Institute, Braga, Portugal (39 ± 9 years old, range 27 to 56 years old; 49% were males). Inclusion criteria for HIV^+ individuals were: infection with HIV-1; receiving HAART for at least 1 year; being regular on HAART compliance (with no history of irregular compliance in the past); plasma viral loads $\leq$50 copies HIV RNA/mL; and baseline $CD4^+$ T cell counts $\leq$500 cells/μL. Information regarding patient's gender, hepatitis C virus (HCV) co-infection, HAART compliance, baseline $CD4^+$ T cell counts, Nadir value and actual $CD4^+$ T cell counts was collected by patients' physician. $CD4^+$ T cell counts were obtained by a reference laboratory. $CD4^+$ T cell count progression for each individual were calculated by subtracting the baseline $CD4^+$ T cell counts (immediately before HAART initiation) from the actual $CD4^+$ T cell counts. The $CD4^+$ T cell slopes (b1) for each individual during the first 12 months of HAART were calculated by the least square estimation method using MO Excel ($CD4^+$ T cell count = b0+b1 ×time; b0 being the CD4+ T cell counts at 0 months of HAART); analysis was restricted to those subjects who had at least three $CD4^+$ T cell measurements during the first year after HAART initiation.

Flow cytometry

All samples were processed for flow cytometric analysis on the day the blood was collected. To stain for cell surface molecules 100 μL of whole blood were incubated with a defined set of antibodies for 15 min at room temperature, followed by 15 min with FACS Lysis Buffer (BD Biosciences), washed and acquired. To determine the expression of the intracellular marker FOXP3, 2 million peripheral blood mononuclear cells (PBMCs), obtained from heparinized blood by Histopaque 1077 (Sigma-Aldrich) gradient centrifugation, were stained for cell surface markers for 20 min, washed, fixed, permeabilized and stained using the FOXP3 Staining Buffer Set (eBiosciences). Antibodies used were anti-CD4 (clone RPA-T4; BD Biosciences), anti-CD3 (clone OKT3 or UCHT1), anti-CD45RO (clone UCHL1), anti-HLA-DR (clone L243), anti-CD127 (clone PHCD127), anti-CD25 (clone BC96, all from Biolegend) and anti-FOXP3 (clone PCH101, eBiosciences). Optimal concentration was determined for each antibody by testing serial dilutions. All samples were acquired on a BD LSR II flow cytometer using FACS DIVA software (Becton and Dickinson, NJ, USA) and data were analysed using FlowJo Software (Tree Star, OR, USA).

Statistical analysis

The normality assumption for parametric tests was tested using the Kolmogorov-Smirnov test (with Dallal-Wilkinson-Lilliefor Significance Correction); since the Tregs percentages from HIV^+ individuals did not follow a normal distribution all tests applied were non-parametric. Groups' medians, variances and proportions were compared using the Mann-Whitney, Levene's and Chi-square tests, respectively. Spearman's rank correlation coefficient was performed to assess the correlation between two variables. P-values less than 0.05 were considered statistically significant.

Results and Discussion

Heterogeneous distribution of Tregs percentages among HIV^+ individuals under HAART

Human Tregs were first identified on the basis of their high-level expression of CD25 (the IL-2Rα chain) [40,41,42] and subsequently by the expression of the Forkhead-box transcription factor FOXP3 [43,44]. However, further human studies have shown that activated $CD4^+$ T cells also up-regulate the expression of CD25 and can transiently express FOXP3 [45,46]. More recently, it was shown that Tregs express low levels of CD127 (the IL-7Rα chain), and therefore this molecule is considered useful as an additional marker to identify this population [47,48,49,50]. Even though the utility of these, and other putative Tregs markers, is still debated, they currently represent the best available markers to identify this cell subset. With this in mind, we chose to identify Tregs within $CD4^+$ T cells ($CD3^+CD4^+$) as the $CD127^{low}CD25^{high}FOXP3^+$ population (Figure 1A).

Using this gating strategy to define Tregs the vast majority of our control individuals (75%) featured Tregs percentages between 5% and 10% (Figure 1B), frequencies similar to those reported by other groups using the same markers and gating strategy [51]. By comparing the median Tregs percentages no differences were

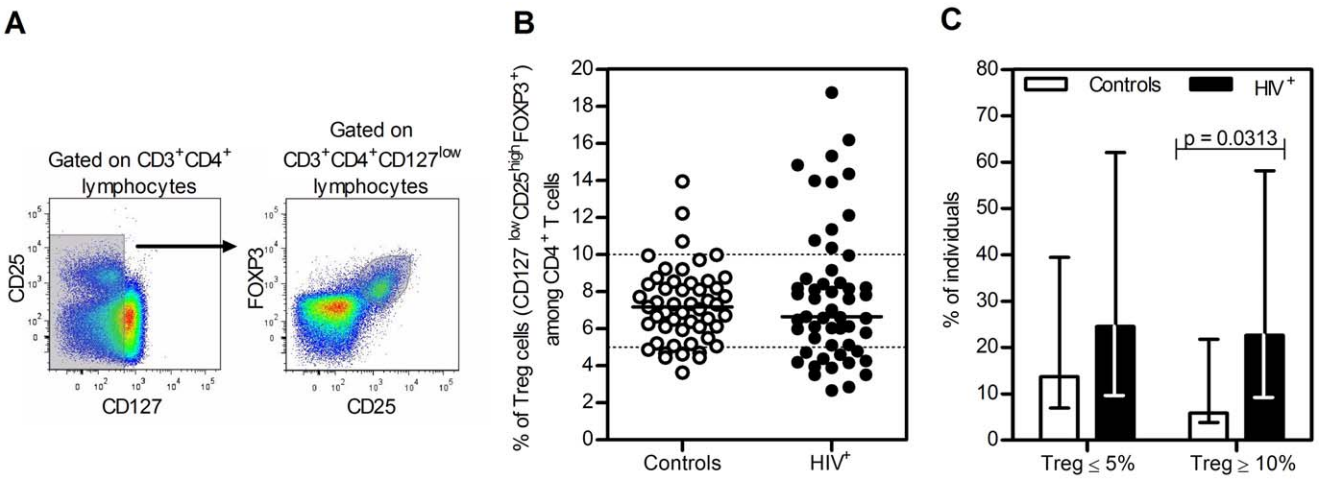

Figure 1. High variability of Tregs percentages in HIV$^+$ individuals. A. Representative dot plots from an HIV$^+$ individual illustrating the gating strategy for Tregs analysis. Lymphocytes (selected according to FSC and SSC) were gated on CD3$^+$CD4$^+$ cells, on low or no expression of CD127 and on the expression of FOXP3 and high levels of CD25. **B.** Tregs percentages amongst CD4$^+$ T cells in control and HIV$^+$ individuals. Each dot represents a single individual and the lines the median Tregs percentages within CD4$^+$ T cells. Dashed lines represent the range of Tregs percentages among total CD4$^+$ T cells described for healthy individuals (between 5% and 10%) [51]. **C.** Percentage of individuals with Tregs ≤5% and ≥10% in controls and HIV$^+$ individuals. P-value for the comparison of these proportions (Chi-square test) and the 95% confidence interval are depicted.
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observed between the HIV$^+$ and control individuals ($p = 0.8250$, Mann-Whitney test; Figure 1B). However, the range of Tregs percentages observed was higher amongst HIV$^+$ individuals ($p = 0.0020$, Levene's test; Figure 1B). It was interesting also to note that the number of individuals with Tregs percentages ≥10% was significantly higher in the HIV cohort as compared to control ($p = 0.0313$, Chi-Square test; Figure 1C).

As studies of HAART-treated HIV$^+$ individuals have yielded conflicting data regarding Tregs percentages [22,23,35,36,37,38,39], we sought to understand if other variables could be influencing this parameter. We found that neither individual's age, number of years in therapy, overall immune activation or infection by the hepatitis C virus (HCV) impacted upon Tregs percentage in our HIV population (Figure S1).

Overall, whilst there were no differences in the median percentage of Tregs in HIV$^+$ and control individuals, there was a wider distribution of Tregs percentages among HIV$^+$ individuals, ranging from 3% to 19%. Furthermore, Tregs frequencies were not related to age, number of years of treatment, overall immune activation or infection by HCV (Figure S1).

The majority of HIV$^+$ individuals with high Tregs percentages featured low Nadir value and incomplete CD4$^+$ T cell recovery

To evaluate whether distinct Tregs percentages were related to different degrees of CD4$^+$ T cell recovery amongst HAART-treated HIV$^+$ individuals, we assessed potential correlations between distinct parameters to evaluate CD4$^+$ T cell reconstitution and Tregs percentages. As shown in Figure 2A, we found no correlation between Tregs percentages and CD4$^+$ T cell counts, CD4$^+$ T cell progression or CD4$^+$ T cell slope ($R = -0.2130$ and $p = 0.1270$; $R = -0.2409$ and $p = 0.0823$; $R = -0.1836$ and $p = 0.2633$, respectively, Spearman's correlation). While there is no clear correlation between the CD4$^+$ T cell reconstitution and Tregs percentage, as previously reported by others [37,39], it is interesting to note that amongst HIV$^+$ individuals with high Tregs proportions (≥10%) the majority featured low CD4$^+$ T cell numbers (<500 cells/μL), low CD4$^+$ T cell recovery and low CD4$^+$ T cell slope (Figure 2A).

To further dissect the link between low CD4$^+$ T cell counts and high Tregs percentages in HIV$^+$ individuals we re-assessed the relationship between these two variables taking into account the gender, number of years on treatment and the Nadir value, all of which are factors known to influence immune reconstitution [3,4,5,15,52,53]. No clear correlations were observed between the Tregs percentages and CD4$^+$ T cell counts when patient's gender ($R = -0.2480$ and $p = 0.1090$ for males; $R = -0.2480$ and $p = 0.4920$ for females, Spearman's correlation), years of therapy ($R = -0.0772$ and $p = 0.7534$ for <5 y HAART; $R = -0.2598$ and $p = 0.1379$ for ≥5 y HAART, Spearman's correlation) and the Nadir value ($R = -0.07719$ and $p = 0.0648$ for <200 cells/μL, $R = 0.0,0963$ and $p = 0.6893$ for ≥200 cells/μL, Spearman's correlation) were taken in consideration (Figure 2B).

Of notice, we observed that almost all individuals with Tregs percentage ≥10% and CD4$^+$ T cell counts ≤500 cells/μL had Nadir values <200 cells/μL (Figure 2B). Since the Nadir value has been considered one of the key factors influencing immune reconstitution [3,4,5], we considered relevant to further explore this association. To do so we analysed CD4$^+$ T cell counts in HIV$^+$ individuals divided on the basis of their Nadir values (<200 and ≥200 cells/μL) and subdivided according to their Tregs frequency (<10%, Figure 3A). As previously reported [3,4,5], we observed that individuals with low Nadir values (<200 cells/μL) had lower CD4$^+$ T cell counts upon treatment ($p = 0.0234$, Mann-Whitney test, Figure 3A). Interestingly, this difference lost statistical significance when only those individuals with Tregs percentages <10% were analysed ($p = 0.1934$ by Mann-Whitney test, Figure 3A).To address the influence of Tregs percentages on the well-established correlation between CD4$^+$ T cell recovery and Nadir value [3,4,5], a correlation between these two variables separating the individuals according to the Tregs percentages was performed. As shown previously by others we observed that CD4$^+$ T cell counts positively correlate with the Nadir value when all HIV$^+$ individuals are considered ($R = 0.4481$ and $p = 0.0008$, Spearman's correlation; Figure 3B). Of interest this correlation

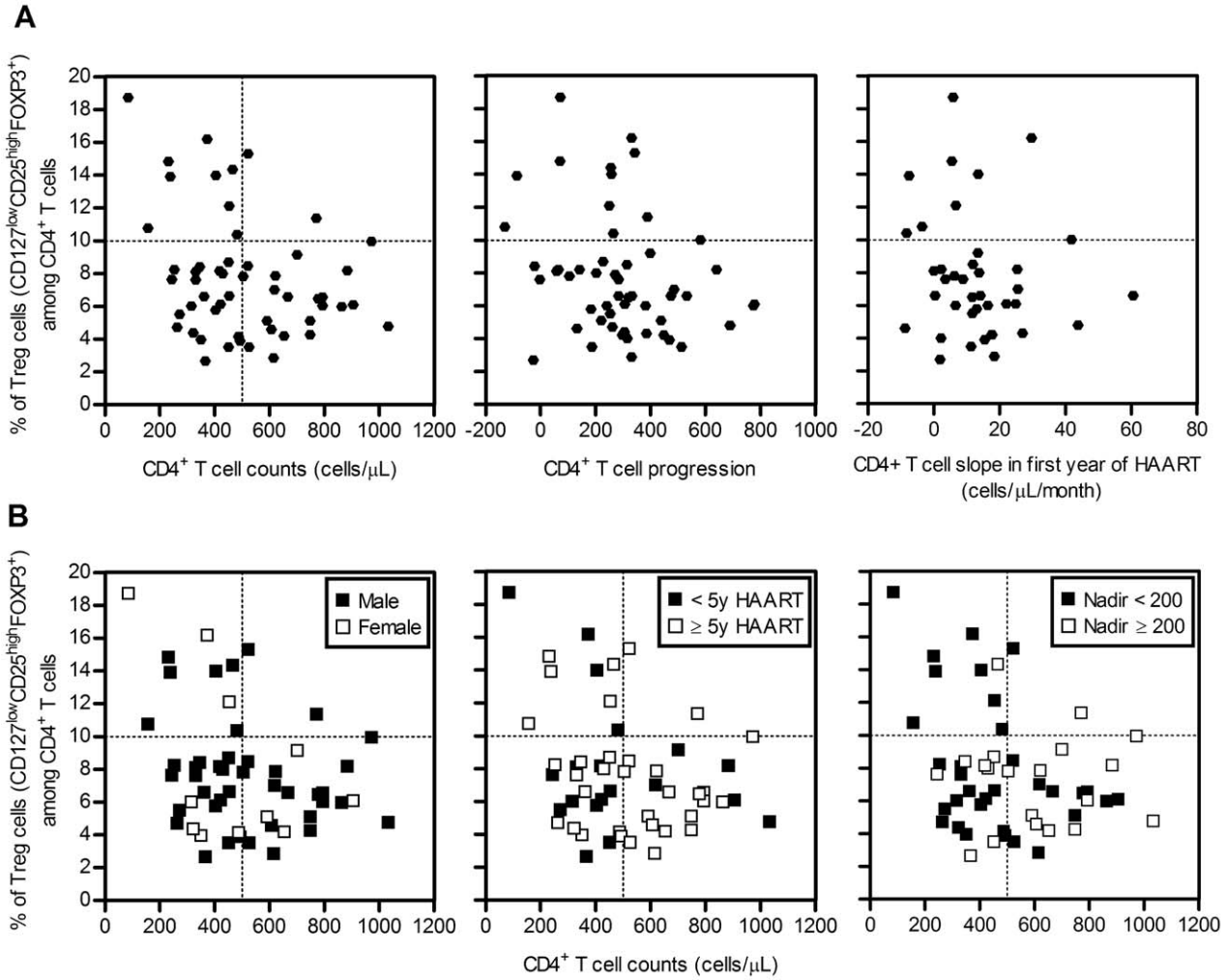

Figure 2. HIV$^+$ individuals with high Tregs percentages feature low CD4$^+$ T cell counts and Nadir values. A. Percentages of Tregs amongst HIV$^+$ individuals were plotted against CD4$^+$ T cell counts, CD4$^+$ T cell progression and CD4$^+$ T cell slope (for the first year of HAART). Each dot represents a single individual. **B.** Tregs percentages were plotted against CD4$^+$ T cell counts in HIV$^+$ individuals grouped by gender) number of years under HAART and the Nadir value.
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was much stronger when only those individuals with ≥10% Tregs were taken into account ($R = 0.8392$ and $p = 0.0006$ by Spearman's correlation; Figure 3B) indicating that CD4$^+$ T cell recovery of individuals with low Nadir is hampered by high proportions of Tregs.

Results reported by Gaardbo *et al.* [36], who analysed a correlation between CD4$^+$ T cell counts and Tregs percentages in patients sub-divided according to baseline CD4$^+$ T cell counts (< and >200 cells/μL), did not find any differences. Their analysis focused on HIV$^+$ patients with over 2 years of treatment. Since we have observed that time in treatment seems to have no impact on the CD4$^+$ T cell counts *vs.* Tregs proportion relation, the discrepancy is most likely due to the use of different markers to define Tregs or to the fact that they used baseline CD4$^+$ counts instead of Nadir values.

Strong correlation between Tregs percentages and CD4$^+$ T cell counts progression in immunologically non-responders HIV$^+$ individuals

While the observation that some individuals are unable to reconstitute the CD4$^+$ T cell numbers to normal values, even after several years of therapy and suppression of viral replication, there is still a lack of consensus on the definition of immunological non-responder individuals [7]. The most well accepted definition for immunological non-responders patients are the ones whose CD4$^+$ T cell counts remained below a threshold (from 350 to 500 cells/μL) after a variable period of time of treatment (from 4 to 7 years) [3,4,5]. Considering as immunological non-responders the individuals under regular HAART for at least 5 years and whose CD4$^+$ T cell counts were <500 cells/μL (14 out of 53 individuals in our population), we observed a strong correlation between Tregs percentages and CD4$^+$ T cell progression ($R = -0.7765$ and $p = 0.0004$, Spearman's correlation, Figure 4), which strengthens the observed association between high Tregs percentage and poor CD4$^+$ T cell reconstitution.

The biological processes that could lead to different Tregs percentages in HIV$^+$ individuals remain unclear. Whilst some studies have shown that Tregs are permissive to HIV infection *in vitro* [19,20], which could lead to the depletion of this subset in HIV$^+$ individuals, others have shown that HIV selectively promotes Tregs survival by reducing apoptosis levels in this subset, and thereby increasing their proportion within the CD4$^+$ T

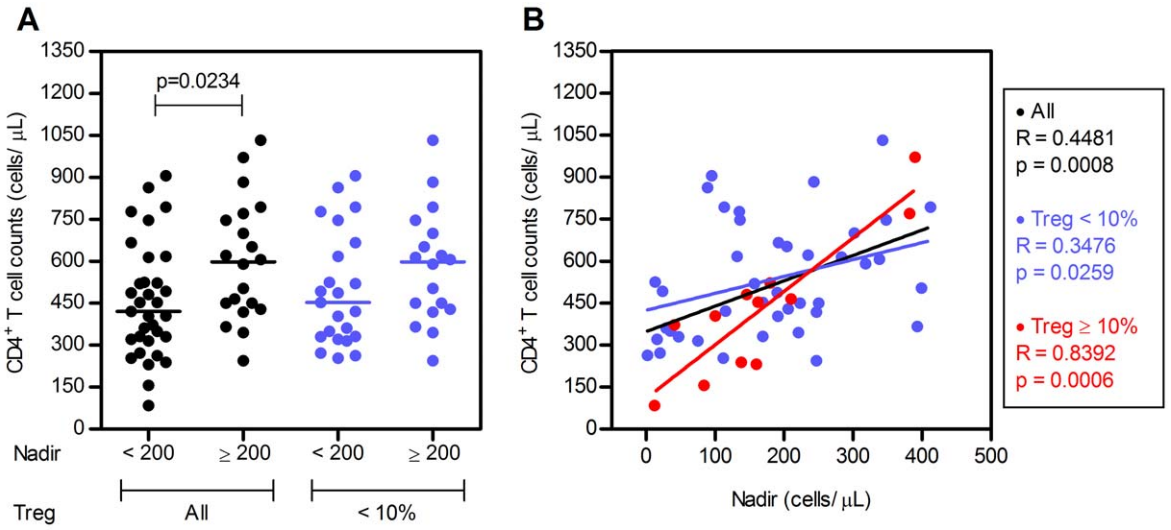

Figure 3. Individuals with high Tregs percentages feature the strongest correlation between CD4$^+$ counts and Nadir values. A. HIV$^+$ individuals were subdivided according to their Nadir counts (<200 and ≥200 cells/µL) and grouped according to Tregs percentages (all individuals and individuals with <10% Tregs) and the CD4$^+$ T cell counts are shown for each group. Each dot represents a single individual and the line the median value for each group. P-value for the comparison of the CD4$^+$ T cell counts is indicated (Mann-Whitney test). **B.** Scatter plots illustrating the correlation between Nadir value and CD4$^+$ T cell counts during HAART. Each dot represents a single individual. P-value for the correlation between CD4$^+$ T cell counts and the Nadir value is shown (Spearman's correlation).
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cell pool as a whole [21]. Several hypotheses have been put forward in order to explain the observed differences in Tregs percentages, such as increased Tregs thymopoiesis [37] or even conversion of conventional CD4$^+$ T cells to Tregs [54,55]. Further studies are still needed in order to fully understand Tregs kinetics during immune reconstitution in HAART-treated HIV$^+$ individuals.

Altogether, our data show that high proportions of Tregs during treatment in individuals who reached low Nadir values (<200 CD4$^+$ T cells/µL) have a synergistic/cumulative negative association with incomplete CD4$^+$ T cell recovery. While the clear association of high percentage of Tregs with low Nadir and incomplete reconstitution would suggest a potential negative impact of Tregs on CD4$^+$ T cell recovery, it cannot be excluded the possibility that Tregs, in individuals with incomplete immune reconstitution, play an important role preventing excessive expansions of oligoclonal populations. This could be of importance to better decide when to start treatment. If during the course of HIV infection an individual already has a high Tregs percentage, our results would support the need for HAART initiation even if CD4$^+$ T cells counts remain relatively high. Further studies involving longitudinal follow up are needed in order to fully understand how the high percentage of Tregs abrogates the CD4$^+$ T cells recovery. Moreover, these studies could also help defining the parameters that synergise with high Tregs frequencies and low Nadir for sub-optimal T cell recovery.

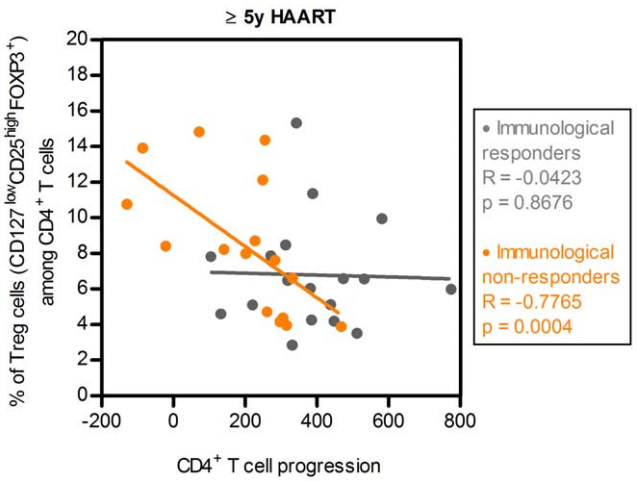

Figure 4. Immunological non-responders present a strong correlation between Tregs percentages and CD4$^+$ T cell progression. Scatter plot illustrating the correlation between Tregs percentages and CD4$^+$ T cell progression. Patients were selected on the basis of being under HAART for at least 5 years and whose actual CD4$^+$ T cell counts are <500 cells/µL or >500 cells/µL. Each dot represents a single individual.
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Supporting Information

Figure S1 Tregs percentages are not affected by HCV infection, age, time in treatment or immune activation. A. Tregs percentages were compared in HIV$^+$ individuals when divided according to co-infection with HCV ($p = 0.1250$, Mann-Whitney test). **B.** Relationship between the Tregs percentages and age (left panel; $R = -0.1520$, $p = 0.2773$; Spearman's correlation), years in therapy (middle panel; $R = -0.0682$, $p = 0.6277$; Spearman's correlation) and overall immune activation (right panel; $R = 0.1400$, $p = 0.3660$; Spearman's correlation). Each dot represents a single individual.
(TIF)

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Author Contributions

Conceived and designed the experiments: AH AGC MC-N. Performed the experiments: AH PA-M CN VC-T PB-S. Analyzed the data: AH PA-M CN VC-T AGC MC-N PC. Contributed reagents/materials/analysis tools: AH SB RS-C AGC MC-N. Wrote the paper: AH PA-M CN AGC MC-N.

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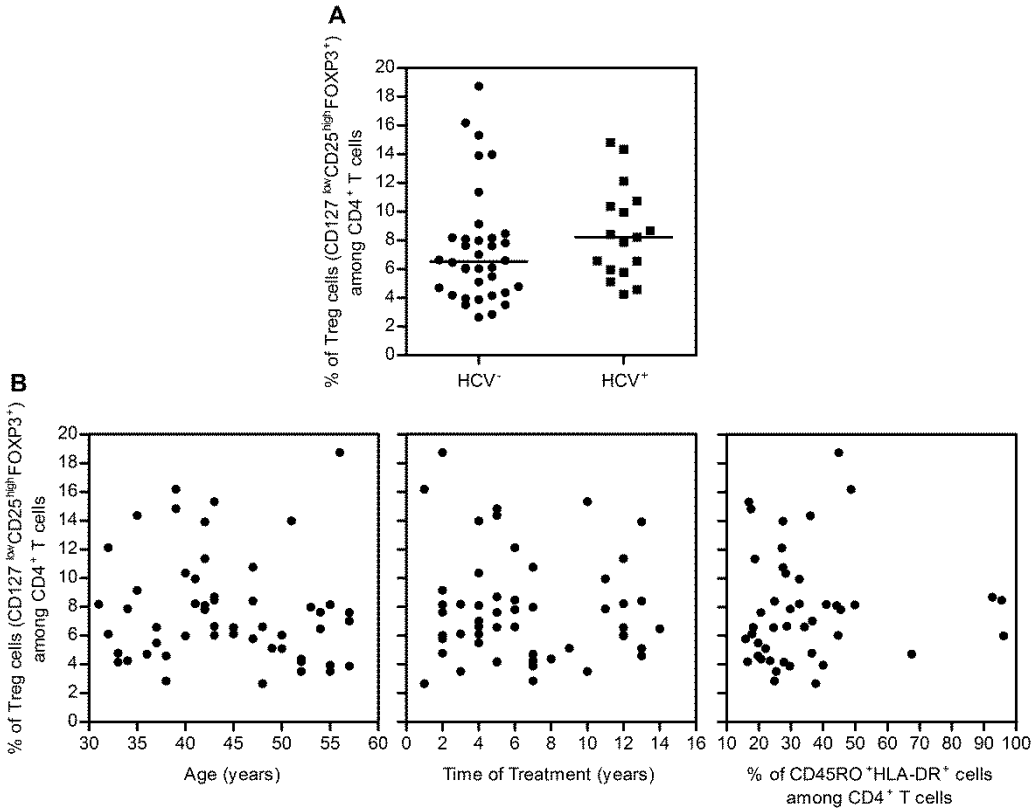

Figure_S1: **Treg percentages are not affected by HCV infection, age, time in treatment or immune activation. A.** Treg percentages were compared in HIV$^+$ individuals when divided according to co-infection with HCV (p=0.1250, Mann -Whitney test). **B.** Relationship between the Treg percentages and age (left panel; R =0.1520, p = 0.2773; Spearman's correlation), years in therapy (middle panel; R =0.0682, p = 0.6277; Spearman's correlation) and overall immune activation (right panel; R = 0.1400, p = 0.3660; Spearman's correlation). Each dot represents a single individual.

2.3. The Dynamics of Regulatory T Cells on the Immune Recovery of Individuals Infected by the Human Immunodeficiency Virus on Antiretroviral Therapy

Claudia Nobrega[1,2,*], Ana Horta[1,2,3,*], Ana Ribeiro[1,2], Vítor Coutinho-Teixeira[1,2], Ana Baldaia[1,2], Catarina L. Santos[1,2], Rui Sarmento-Castro[3], Margarida Correia-Neves[1,2]

1 Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal.

2 ICVS/3B's, PT Government Associate Laboratory, Braga/Guimarães, Portugal.

3 Infectious Diseases Service of the Centro Hospitalar do Porto, Hospital Joaquim Urbano Unit, Porto, Portugal.

* These authors contributed equally to this paper

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2.3.1. Abstract

Background: The introduction of highly active antiretroviral therapy (HAART) to treat individuals infected by the human immunodeficiency virus (HIV) resulted in a tremendous decrease in morbidity and mortality. Nevertheless, a sizeable percentage of HIV-infected individuals on HAART fail to increase their $CD4^+$ T cell counts, despite showing viral load suppression. Several factors have been put forward to explain this immune reconstitution impairment. Among them, the effect of regulatory T cells (Treg) has been widely discussed, although the dynamics and the role of Treg remain controversial.

Methods: A longitudinal study on 81 HIV-infected patients was performed. All individuals had criteria to initiate HAART, and were followed from the moment they initiated HAART (baseline) and during the following 24 months. $CD4^+$ T cell counts, Treg percentages and specific Treg subpopulations (naïve and cycling cells) were evaluated.

Results: Treg percentages from baseline and up to 6 months of HAART are higher in comparison to healthy controls, mainly for the individuals with <200 $CD4^+$ T cells/μL at baseline. Of notice there is great diversity on the values, particularly for this last group of individuals. Despite the evolution of Treg percentages to levels similar to healthy controls, the percentage of $CD45RA^+$ (naïve) and of $Ki67^+$ (cycling) cells among Treg remained altered throughout the 24 months of follow-up.

Conclusions: The fact that some patients reach very low $CD4^+$ T cell counts before HAART initiation leads to a deregulation of the Treg subpopulations that are not fully recovered, even after 24 month of efficient HAART. These patients are not just unable to recover the $CD4^+$ T cells to numbers considered normal but in addition the quality of the reconstitution is also jeopardize.

Running title (26/40 characteres): Treg dynamics during HAART.

Keywords: Human Immunodeficiency Virus; Antiretroviral therapy; Immune reconstitution; Regulatory T cells.

2.3.2. Introduction

During the course of the infection by the human immunodeficiency virus (HIV) various mechanisms contribute to the gradual depletion of $CD4^+$ T cells. The deleterious effects and immune dysfunction are extended to other cells of the immune system. As a result, a steady imbalance occurs mainly at the level of the cell-mediated immunity termed as acquired immunodeficiency syndrome (AIDS). If left untreated, AIDS will lead to a final stage of inability to counteract major life threatening infections and some tumors, resulting in high morbidity and mortality rates [1].

The emergence of drugs with potent antiretroviral activity, and their use in combination for the treatment of HIV infection markedly reduced the negative effects of HIV infection. The highly active antiretroviral therapy (HAART) dampens viral replication for the vast majority of the patients with a consequent increase of the $CD4^+$ T cell counts, culminating to what is called immune reconstitution [2]. However, among the HIV-infected individuals that initiate HAART and become virologically suppressed during several years (4 to 7 years), 15 to 40% of them (number that varies depending on the study) fail to reconstitute the $CD4^+$ T cell counts. These individuals are referred to as immunological non-responders [3].

The failure of the immune system to recover might result from insufficient production and/or excessive destruction of $CD4^+$ T cells. Several conditions may contribute, individually or in combination, to this defect such as [3-5]: failure of the bone marrow to produce hematopoietic stem cells; impaired thymic function; advanced age; immunosenescence; residual viral replication; infection by more cytopathogenic virus as those that use CXCR4 as a co-receptor; enhanced T cell activation and apoptosis; gut microbial translocation that could lead to increased T cell activation and destruction; or co-infections by hepatitis C virus, cytomegalovirus, or other herpes virus. It is worth noticing that some of these conditions can be associated to each other, being one the cause or effect of the others. Low $CD4^+$ T cell counts at the therapy onset has consistently been shown as a strong predictor for worse $CD4^+$ T cell counts recovery even for virologically suppressed patients undergoing HAART [6-8].

The role of regulatory T cells (Treg) in HIV infection pathogenesis has been extensively debated [9]. These cells may play a beneficial role by dampening the immune activation [10-12] and suppressing HIV-replication within conventional $CD4^+$ T cells [13]. On the other hand, Treg may have a harmful role by impairing specific anti-HIV immune response [14-17], inhibiting

lymphopenia-induced proliferation [18, 19], contributing for gut microbial translocation [20, 21] and for an exacerbated fibrosis of lymphoid tissues [22].

Most of the studies describe that Treg percentage among $CD4^+$ T cells in chronic HIV-infected patients naïve for HAART is higher in comparison to healthy controls [23-28], though some studies observe no differences [10, 14]. The evolution of Treg percentage upon HAART initiation is even more controversial; despite that most studies describe a decrease in the Treg percentage among individuals receiving HAART [24-26, 28-30], many claim that these values are maintained above the ones considered normal from healthy controls [24, 26, 27, 29]. The lack of consensus in this aspect might be related to different factors, namely differences on the study groups characteristics (cross-sectional over longitudinal studies; patients heterogeneity over homogeneity at HAART onset; differences on the size of the cohorts analyzed, being most of them relatively small) and/or from different strategies used to identify Treg.

The effect of Treg percentages on the immune recovery of patients on effective HAART has also been explored. Despite high Treg percentages have been related to an impaired immune reconstitution [18, 31-34], none of these studies have correlated the Treg percentage at HAART onset with immune recovery. To better understand the temporal evolution of the Treg (percentages and subpopulations) during HAART and the potential effect of Treg percentages, at baseline and during therapy, on the immune reconstitution, we evaluated longitudinally 81 HIV-infected individuals since the moment they initiated HAART and for the following 24 months.

2.3.3. Materials and Methods

Ethics Statement

Individuals undergoing medical care at the Hospital Joaquim Urbano Unity of Centro Hospitalar do Porto, Porto, Portugal (HJUU/CHP) were selected for this study. After explanation of the entire study protocol, all the patients signed an informed consent prior to their enrollment in the study. A local Ethical Committee approval was received for this study (reference 168/CES on 2nd October 2009). It should be noted that HJUU/CHP is the one that follows a great proportion of the HIV infected individuals from the city of Porto and surrounding as its main focus is on infectious diseases.

Study Population

Individuals were enrolled in this study with the following criteria: age over 18 years; chronically infected by HIV-1 (from now on referred simply as HIV); naïve for HAART but with criteria to initiate HAART. The moment of HAART initiation is referred in the study as 0 months or as baseline. The individuals were followed thereafter at 2, 6, 9, 12, 16, 20 and 24 months. HAART schemes chosen (Table 1) for each individual took into consideration the scientific policy, national and international guidelines [35], particular characteristics of each individual and drug cost, as it is usual for all individuals being followed at our care unit.

At the end of the 24 months follow-up, only those patients that had been regular on therapy compliance and had plasma viral loads bellow 50 copies/mL, were included in this study in a total of 81 (demographic features depicted in Table 1). From these, two individuals had a virological blip at 24 months despite being regular on HAART; these individuals were maintained in the study after confirming virological suppression at 28 months (all virological blips were under 200 copies/mL; Figure S1). In two patients, the data relative to 28 months of follow-up were used instead of 24 months (one has missed the 24 months visit and the other patient had pneumonia at 24 months, AH031 and AH030, respectively, in Supplementary Table 1). In parallel, we enrolled a group of age- and gender-matched HIV-uninfected subjects (Table 1).

Sample processing and flow cytometry

In each study visit blood was collected and sent both for routine analysis by a reference laboratory ($CD4^+$ T cell counts and HIV viral load) and for complementary multiparametric flow cytometry analysis. These procedures were performed on the same day the blood was collected.

For the multiparametric flow cytometry surface staining, 100µL of whole blood was incubated for 15 min, at room temperature, with a defined set of monoclonal antibodies directed to human molecules (all from Biolegend): Pacific anti-CD3 (clone OKT3), anti-CD4 (clone RPA-T4), anti-CD8 (clone RPA-T8), anti-CD45RA (clone HI100), anti-CD45RO (clone UCHL1) and anti-HLA-DR (clone L243). Afterwards, erythrocytes were lysed upon incubation for 15 min in FACS Lysis Buffer (BD Biosciences, San Jose, CA, USA), at room temperature. Cells were washed with FACS buffer, and acquired on a flow cytometer.

The analysis of Treg was performed in peripheral blood mononuclear cells (PBMCs) isolated upon gradient centrifugation with Histopaque 1077 (Sigma-Aldrich). Two million PBMCs were first labeled with the surface markers for 15 min at room temperature: anti-CD4, anti-CD25

(clone BC96), anti-CD45RA, anti-CD127 (clone AO19D5, all from Biolegend) and anti-CD3 (clone UCHT1, BD Horizon). After the excess antibody was washed out, cells were fixed and permeabilized using the FOXP3 Staining Buffer Set (eBioscience, San Diego, CA, USA), accordingly to the manufacturer's instructions. Cells were afterwards incubated for 30min with anti-FOXP3 (clone PCH101, eBiosciences) and anti-Ki67 (clone MOPC-21, BD Pharmigen). Cells were washed and acquired on the flow cytometer.

Variable	HIV-infected (n=81)	Control (n=44)
Age, years (mean, range) [1]	41 (22-67) [2]	39.5 (22-56)
Male gender (%, n)	77.8% (63) [3]	68.0% (30)
HIV transmission mode (n)		
Intravenous drug users [4]	19	NA
Men who have sex with men	23	
Heterosexuals	37	
Unknown	2	
$CD4^+$ T cell counts, cells/μL (mean, range) [1]	274 (8-1033)	NA
log_{10} viral load, copies/mL(mean, range) [1]	5.33 (3.8-7.0)	NA
With criteria for AIDS (%, n) [5]	44.4% (36)	NA
Hepatitis C virus-positive (%, n) [6]	22.2% (18)	NA
Hepatitis B virus-positive (%, n)	1.2% (1)	NA
With viral mutations of resistance (%, n)	18.5% (15)	NA
HAART regimen components (n)		
2 NRTIs		
TDF+FTC	61	NA
ABC+3TC	19	
ZDV+3TC	1	
3rd Drug (NNRTI or PI/r)		
EFV/NVP	68/1	
DRVr/LPVr	10/2	

Table 1. Demographic information of the study individuals.

[1]At baseline; [2]No significant difference between the mean age of the two groups (unpaired T-test; p=0.533); [3]No significant difference between the percentage of males on the two groups (Fisher exact probability test; p=0,285); [4]Five of these patients presented also heterosexual risk for HIV transmission; [5]Accordingly to the guidelines of Centers for Disease Control and Prevention [36]; [6]Positive for hepatitis C virus antibodies and RNA.

Abbreviations: ABC, abacavir; DRVr, ritonavir boosted darunavir; EFV, efavirenz; FTC, emtricitabine; LPVr, ritonavir boosted lopinavir; NA, not applicable; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside (or nucleotide) analogue reverse transcriptase inhibitors; NVP, nevirapine; PI/r, ritonavir boosted protease inhibitor; TDF, tenofovir disoproxil fumarate; ZDF, zidovudine; 3TC, lamivudine.

Each of the antibodies used were titrated using serial dilutions until the optimal concentrations were determined. All samples were acquired on a BD LSRII flow cytometer using FACS DIVA software (Becton and Dickinson, NJ, USA) and data were analyzed using FlowJo Software (Tree Star, OR, USA).

Statistical analysis

To evaluate normal distribution of the variables, skewness and kurtosis values were calculated and approximate normal distribution was defined for variables with absolute values of skewness below 3 and kurtosis below 8 [37].

As all variables followed a normal distribution, parametric tests were performed: Pearson's correlation coefficient test to relate two distinct variables; unpaired T-test to compare two group's means; one-way ANOVA followed by Bonferroni's multiple comparison tests to compare more than two independent groups' means; ANOVA repeated measurements (using the Geisser-Greenhouse correction for sphericity) followed by Bonferroni's multiple comparison tests to evaluate progression; and two-way ANOVA repeated measurements followed by Sidak's multiple comparison test to compare and evaluate progression of more than two independent groups of individuals. To evaluate the predictors of a specific event, hierarchical linear regression models were used. Overall, significance was assumed when $p<0.05$ and represented in the graphs as *.

To perform repeated measurements analysis, missing values are not allowed; to overcome this limitation (and only to perform these statistical analysis in Figures 1C, 1D and 5), these values were estimated by calculating the mean of the neighbor values (estimated values represented 1.25% and 2.89% of all the $CD4^+$ T cell counts and Treg proportions measurements, respectively). It should be noted that the analysis was also performed excluding subjects who had at least one estimated value and the results were similar to those obtained using estimated values. Supplementary Table 1 depicts the raw data and highlights the values that were estimated.

Statistical analyses were performed using the IBM SPSS v.22 or the GraphPad Prism v.6.

Patient ID	%Treg among CD4+ T cells over time on HAART								CD4+ T cell counts over time on HAART						
	0	2	6	9	12	16	20	24	0	2	6	12	16	20	24
AH001	31,5	6,9	12,0	6,6	7,7	8,8	7,9	6,4	11	59	116	180	301	373	382
AH003	19,5	6,0	12,0	7,5	7,5	7,6	7,0	6,0	22	135	128	186	159	182	191
AH004	11,5	9,3	8,2	10,2	11,9	12,9	8,5	6,5	292	571	460	519	451	467	435
AH005	12,9	5,3	7,3	7,5	7,0	9,3	5,8	6,6	257	413	370	549	604	585	344
AH006		14,0	13,4	15,7	10,2	13,4	11,7	14,8	43	177	274	252	304	367	363
AH007	12,5	9,9	10,8	5,1	11,0	11,8	8,1	11,3	147	414	425	534	819	629	679
AH009	10,9	8,3	5,7	8,7	12,4	1,2	7,4	13,6	310	427	544	555	451	625	600
AH010	9,0	7,4	7,4	6,6	6,8	7,0	7,8	9,6	316	285	325	365	379	675	473
AH011	12,6	13,9	20,3	4,9	16,4	15,3	19,9	11,5	182	176	208	198	220	301	304
AH012	12,2	10,7	13,3	10,4	9,8	10,0	6,5	9,8	230	347	508	472	532	646	641
AH013	2,6	21,7	23,3	19,3	15,2	11,4	10,2	7,8	30	60	190	204	329	273	303
AH014	4,6	4,1	4,7	9,1	5,6	4,4	10,8		334	948	635	639	612	624	751
AH015	6,7	8,7	7,7	6,5	6,3	6,8	4,2	7,3	291	120	111	141	102	134	138
AH016	7,9	9,1	7,9	9,4	9,9	10,6	11,1	9,7	974	1078	1284	1257	1035	1449	1380
AH017	15,4	17,8	14,6	14,5	11,4	12,2	10,8	11,7	408	448	486	524	468	443	374
AH018	12,5	10,3	11,2	10,8	8,7	10,1	9,4	10,5	338	456	576	1042	850	818	747
AH021	12,6	7,9	5,0	7,0	8,9	7,7	4,0	6,4	239	529	746	649	693	665	644
AH022	13,4	11,4	10,7	12,1	12,3	9,8	13,9	11,6	268	321	435	395	344	538	506
AH023	18,0	12,0	10,4	11,5	10,9	8,1	12,0	10,8	314	459	530	626	691	641	658
AH025	12,7	6,5	10,0	8,9	3,2	7,2	6,4	3,0	278	287	201	245	243	288	378
AH026	6,4	6,6	6,6	7,7	3,7	5,0	3,1	1,1	665	1030	655	996	1180	799	1242
AH027	7,5	6,1	5,5	8,0	1,7	4,6	5,5	2,2	415	441	393	658	840	667	972
AH030	5,0	4,2	7,0	4,5	5,1	5,2	5,0	2,3	330	390	419	449	567	519	477
AH031	6,5	8,5	8,9	10,5	8,1	3,9	6,0	7,4	228	323	339	300	345	318	458
AH032	12,1	14,7	12,4	13,7	13,0	12,2	11,5	10,8	303	333	464	400	504	480	452
AH033	7,3	11,6	14,5	17,4	8,7	11,0	6,2	6,7	68	270	282	428	512	515	454
AH034	3,2	3,6	3,8	3,6	2,8	4,6	4,0	3,7	306	400	475	507	392	612	588
AH035	11,1	13,1	10,2	11,5	10,9	11,6	11,4	10,9	25	229	178	285	250	360	299
AH036	7,8	8,7	9,2	8,4	8,2	1,1	8,4	7,8	263	327	330	392	439	494	411
AH037	2,6	12,5	21,6	17,4	12,2	3,6	8,8	6,6	92	163	169	133	322	368	322
AH039	17,3	42,9	42,2	20,9	19,1	22,3	16,6	16,1	9	77	72	100	155	121	152
AH040	10,2	10,4	9,0	12,5	8,0	9,8	11,9	10,4	422	475	329	402	563	398	452
AH041	8,1	11,5	13,6	9,7	7,3	4,8	6,7	8,3	397	674	535	692	692	622	659
AH042	9,6	15,8	13,5	14,4	11,3	12,6	17,1	10,3	262	366	386	426	425	397	538
AH044	0,4	8,5	12,9	14,7	13,7	9,3	9,5	8,6	56	489	463	537	505	434	439
AH045	8,1	7,5	7,0	8,1	5,9	6,5	2,1	6,5	481	771	880	809	817	830	811
AH046	9,9	9,5	9,2	8,5	6,4	5,0	7,3	3,0	388	598	1002	725	890	972	724
AH047	9,4	9,6	9,8	7,5	7,2	6,1	8,6	7,0	321	428	308	363	399	524	486
AH048	22,1	24,9	24,3	20,0	12,1	29,7	17,0	12,4	63	205	126	221	207	236	202
AH049	6,8	8,9	9,6	9,2	11,9	11,6	11,0	8,4	415	420	424	488	599	533	503
AH050	12,9	17,2	16,7	11,9	12,2	10,2	16,3	6,8	303	514	435	389	452	481	581
AH051	7,1	9,3	8,5	7,6	7,0	7,1	8,1	6,3	784	649	852	757	1039	761	998
AH052	8,0	8,4	9,3	5,7	6,5	7,7	7,4	2,2	107	165	213	300	290	360	348
AH053	15,3	20,1	21,1	20,0	9,1	10,4	10,0	10,7	32	244	256	518	475	526	337
AH054	7,6	8,5	9,3	6,5	8,2	7,2	6,3	6,7	286	505	579	747	652	477	581
AH058	10,1	12,8	12,6	13,7	10,2	7,5	13,3	8,0	326	272	295	414	385	406	373
AH059	5,3	5,6	6,8	6,4	2,6	7,4	0,7	7,4	1033	872	960	847	895	888	933
AH060	5,1	15,8	10,8	11,0	11,7	9,3	0,7	6,3	341	516	524	872	614	864	629
AH061	16,9	5,5	5,8	6,3	4,2	5,6	3,8	5,7	339	410	398	398	537	546	642
AH062	15,5	10,0	10,4	10,8	10,0	9,3	2,5	8,3	205	391	361	444	473	565	512
AH063	13,4	8,7	7,9	6,8	7,6	8,3	4,9	7,6	146	262	293	248	335	358	300
AH064	11,8	13,2	10,7	10,7	10,0	10,2	1,4	8,9	603	580	724	772	865	713	694
AH065	12,1	8,7	6,4	8,7	6,9	7,2	5,7	7,0	319	433	673	593	654	708	716

AH066	29,4	48,6	14,9	9,9	14,7	14,1	10,1	9,6	12	112	155	209	274	475	375
AH067	10,4	8,8	5,2	7,6	5,5	6,1	5,6	5,1	504	631	744	791	857	968	950
AH068	9,6	6,9	5,1	5,2	6,7	7,5	6,5	7,4	540	680	887	1093	866	911	1097
AH069	11,3	9,9	21,5	8,4	5,8	6,6	6,7	4,6	334	350	397	546	595	521	624
AH070	8,4	7,4	6,1	4,8	6,1	7,3	3,9	4,3	269	521	574	575	660	659	630
AH073	35,4	23,9	5,9	9,2	9,8	7,7	11,3	11,9	37	85	118	104	106	140	222
AH074	18,1	20,0	21,8	17,9	13,9	9,3	14,1	13,6	61	201	341	354	331	337	380
AH075	7,1	5,3	5,8	4,7	7,2	6,2	5,0	2,1	319	412	608	350	462	468	468
AH076	7,8	8,9	8,6	6,1	8,9	8,3	5,9	11,0	296	352	407	550	604	623	544
AH077	8,9	11,5	13,4	3,6	8,5	7,3	5,3	6,9	471	748	1227	1145	930	1355	1213
AH078	6,6	5,3	5,7	4,4	2,6	5,8	2,3	7,6	208	426	297	422	518	447	412
AH079	15,4	14,3	5,0	8,6	7,9	5,9	8,4	5,7	47	142	166	253	176	226	307
AH080	7,5	11,4	6,3	12,3	10,9	8,3	9,2	9,1	14	167	197	197	263	327	302
AH081	11,2	9,4	1,6	7,2	7,8	1,8	7,7	7,6	181	262	324	320	327	463	467
AH082	14,6	11,0	2,0	6,4	6,8	5,6	6,6	6,1	176	218	272	370	363	347	409
AH083	27,3	16,1	4,0	14,2	12,1	0,8	8,7	13,0	181	325	353	508	467	768	589
AH084	8,0	6,1	10,1	5,3	3,5	2,1	9,2	13,4	243	273	336	332	379	448	387
AH085	17,6	15,1	16,5	12,7	12,1	14,7	11,9		8	43	47	65	119	174	186
AH086	8,6	10,0	10,7	16,4	11,6	10,0	5,1	8,8	92	202	159	159	191	165	214
AH087	1,5	11,6	20,5	10,8	7,0	6,9	8,0	7,7	42	142	188	335	347	414	511
AH088	5,6	13,6	10,1	10,5	9,1	8,1	10,0	9,7	174	227	238	180	241	277	274
AH089	5,1	13,4	3,3	8,3	10,7	8,3	8,9	9,6	143	396	254	328	240	304	263
AH090	16,2	12,2	13,4	7,6	9,3	6,0	6,0	4,9	20	143	165	249	320	404	380
AH091		8,5	9,7	5,4	2,7	7,2	6,7	6,3	97	157	167	192	213	247	218
AH092		11,6	14,3	8,7	3,2	12,0	9,7	10,2	164	461	310	628	401	514	428
AH093		6,7	10,5	0,8	2,4	7,8	3,9	2,0	193	404	602	774	707	766	726
AH095	5,8	7,3	8,2	4,8	9,2	8,5	5,3	5,3	94	308	222	253	266	319	341
AH096	10,1	11,6	18,8	7,1	16,4	26,4	16,9	17,4	216	424	375	550	590	745	692

Supplementary Table 1. Treg percentage among $CD4^+$ T cells and $CD4^+$ T cell counts for each HIV-infected individual enrolled in the study. Estimated missing values are underlined.

2.3.4. Results

Evolution of Treg percentages during HAART varies accordingly to the $CD4^+$ T cell counts at baseline

In our cohort of HIV-infected patients, HAART resulted in a rapid and strong decline of the plasma viral load for all patients (Figure S1) and in an overall increment of the $CD4^+$ T cell counts over time of therapy (Figure S2) in accordance with what has been described for other cohorts following similar HAART regimens [35].

The selection of markers to define Treg is a controversial issue. We chose to identify Treg within $CD4^+$ T cells ($CD3^+CD4^+$) as the $CD127^{low}CD25^{high}FOXP3^+$ cells accordingly to Sakaguchi *et al.* [38] using the gating strategy described before [33]. As previously shown by most of the studies [23-28], Treg percentages are significantly higher in HIV-infected individuals naïve for HAART in comparison to healthy controls. During therapy, Treg percentages tend to normalize for

the majority of the individuals reaching values that are not statistically significant different from healthy controls at about 9 months of HAART (Figure 1A).

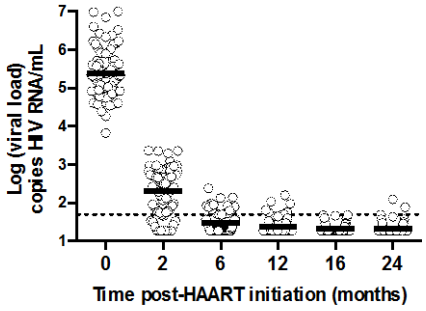

Figure S1. HAART leads to a decrease in the plasma viral load of all treated HIV-infected individuals. Each dot represents a single individual, the horizontal black lines the mean and the horizontal dashed line represents 50 copies of plasma HIV RNA/mL.

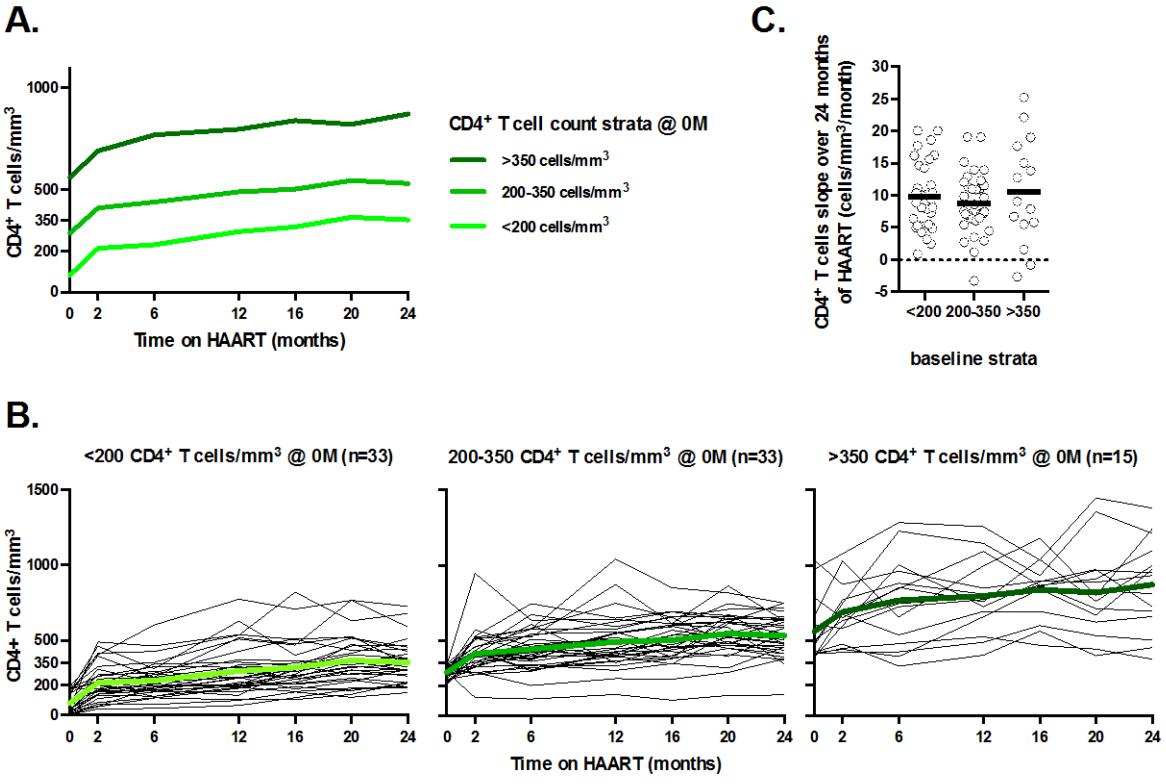

Figure S2. The CD4+ T cell counts rate of increase is similar between individuals with quite distinct CD4+ T cell counts at baseline. A. CD4+ T cell progression for three defined CD4+ T cell strata. The mean of each time-point is represented for the three strata independently. **B.** The progression of the CD4+ T cells counts is represented for each individual independently (black thin lines) and for the mean of all the individuals (thicker a green line). **C.** Comparison of the rates of CD4+ T cell recovery during the 24 months of HAART between the three defined strata. Slopes were calculated by the least square estimation method, as previously described [33]. Each dot represents a single individual and the solid horizontal line the mean. Comparisons were performed using a one-way ANOVA test followed by Bonferroni's multiple comparison tests; no statistical significant differences were observed.

It has been previously described that for HIV-infected HAART-naïve individuals, Treg percentages correlate negatively with the $CD4^+$ T cell counts [39]. This observation is confirmed in the present study (r=-0.3195, p=0.0046; Pearson correlation). To understand whether Treg percentage evolution during therapy differs accordingly to the disease stage at HAART initiation, individuals were stratified based on their $CD4^+$ T cell counts at baseline (<200, 200-350, and >350 $CD4^+$ T cells/μL). Interestingly, the differences observed on the Treg percentages of the overall HIV-infected individuals are only evident for the individuals that initiate HAART at very low $CD4^+$ cell counts (Figure 1B). Due to the high variability on the Treg percentages depicted in Figure 1A and B, we consider that it would be of relevance to show the evolution of Treg percentages for each individual (Figure 1C and D). Overall, we can see a great diversity on the evolution of the Treg percentages (Figure 1C). Nevertheless, a decline in the Treg percentages is observed, that becomes significantly different from the baseline from 12 months of HAART on (Figure 1C). As can be seen in Figure 1D, the evolution of Treg percentages in most individuals with $CD4^+$ T cell counts above 200 is quite similar, with a few individuals showing fluctuations. This is not the case for individuals that initiate HAART with <200 $CD4^+$ T cell/μL; for the majority of these individuals, a high range of Treg percentages is present at baseline and their evolution is also very diverse (Figure 1D).

The role of Treg in the immune recovery process in HIV-infected patients upon effective HAART remains to be elucidated. Some cross-sectional studies with individuals on HAART suggested a negative role of a high Treg percentage on the immune reconstitution during HAART [18, 31-34], but also the contrary has been proposed [41].

To evaluate whether Treg percentages at baseline affects $CD4^+$ T cell reconstitution among HIV-infected individuals under effective HAART, we assess the correlation between Treg percentage at baseline and $CD4^+$ T cell reconstitution. We observed that high Treg percentages at baseline negatively correlate with $CD4^+$ T cell counts at 24 months (Figure 2A). As a result, when HIV-infected individuals were subdivided according to their Treg percentage at baseline (<10% and ≥ 10% as in [41]), those that present higher Treg percentages at baseline, showed lower $CD4^+$ T cell counts at 24 months (Figure 2B). To evaluate whether this difference could be related to the 10% Treg cut-off, the same analysis was performed upon stratification of individuals based on Treg percentages quartiles of or our healthy control group (<5^{th}, 5^{th} to 95^{th} and >95^{th}; upper 5^{th} percentile limit = 4.2%; lower 95^{th} percentile limit = 13.3%) and similar results were found (Figure S3).

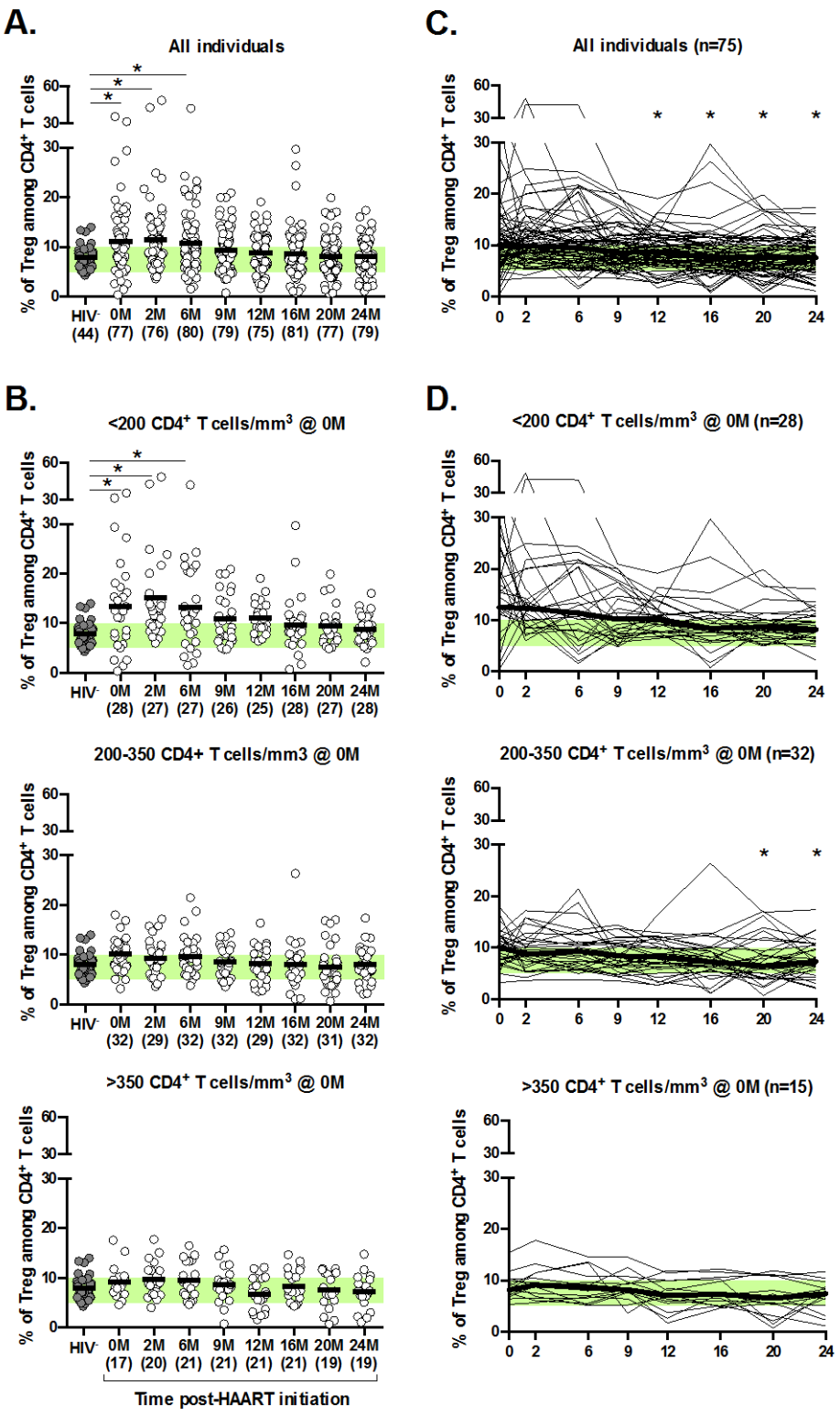

Figure 1. The most immunosuppressed individuals at HAART onset tend to show higher percentages of Treg, which decline with HAART for most patients. A. Overall comparison of the Treg percentages of healthy controls and HIV-infected individuals at different time-points upon HAART initiation. **B**. Comparison of the Treg percentages of healthy controls and HIV-infected patients stratified accordingly to their $CD4^+$ T cell counts at baseline. **C.** Longitudinal evaluation of Treg percentages throughout the first 24 months of HAART. **D.** Stratification of HIV-infected individuals accordingly to $CD4^+$ T cell counts at baseline and longitudinal evaluation of Treg percentages throughout the first 24

months of HAART. For panels A and B, each dot represents a single individual, the horizontal black line the group mean and the shaded green horizontal bar represents the range of Treg percentages among CD4+ T cells described for healthy individuals using similar markers [14]. Comparisons were performed using a one-way ANOVA test followed by Bonferroni's multiple comparison tests using as reference group the HIV-uninfected individuals. For panels C and D, each thin line represents the Treg evolution of a single individual and the bold lines represent the means of the individuals for each time-point. The evolution of Treg percentage with HAART was evaluated using repeated measurements ANOVA test followed by Bonferroni's multiple comparison tests using as reference the HAART-naïve individuals.

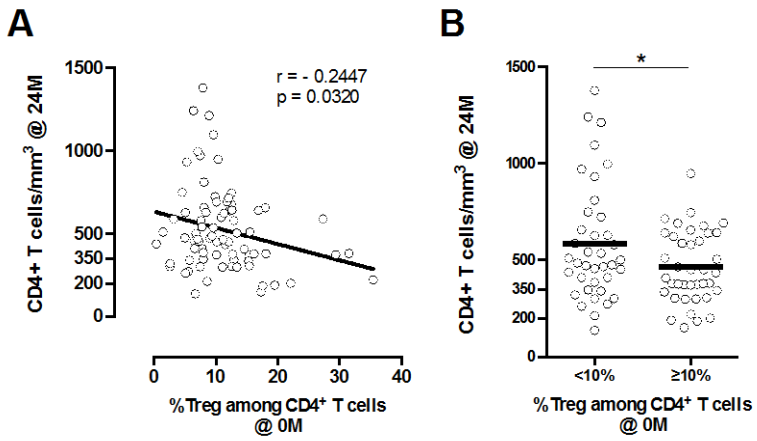

Figure 2. Individuals with higher Treg percentages at baseline have lower CD4+ T cell counts at 24 months. A. Relation between Treg percentage at baseline and CD4+ T cell counts at 24 months of HAART (n=77) by Pearson's correlation. **B.** Comparison of the CD4+ T cells counts at 24 months of HAART between individuals with low (<10%; n=38) and high (≥10%; n=39) Treg percentages at baseline using an unpaired T-test. Each dot represents a single individual and the horizontal line the mean.

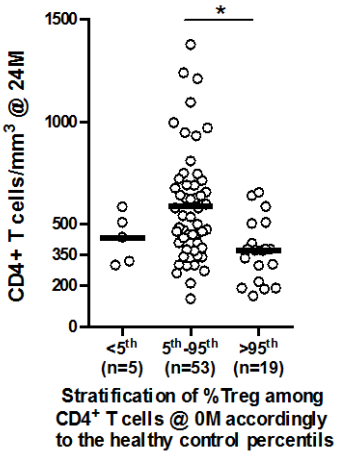

Figure S3. Individuals with higher Treg percentages at baseline have lower CD4+ T cell counts at 24 months. Data on figure 2B was re-analyzed upon stratification of the individuals by percentiles of Treg percentages of the HIV-uninfected group (higher 5th percentile limit of 4.2%; lower 95th percentile limit of 13.3%). Comparisons were performed between the three groups using one-way ANOVA test followed by Bonferroni's multiple comparison tests.

The variation in the CD4+ T cell counts (i.e. the difference between the CD4+ T cell counts at 24 and 0 months) and the CD4+ T cell counts slope in the first 24 months of therapy (calculate by the mean square estimation model, as described in [33]) were independent of the CD4+ T cell counts at baseline (respectively, r=-0.0288, p=0.7887 and r=-0.0086, p=0.9390 by Pearson's correlation) as it has been reported by previous studies [6, 42-44]. To evaluate whether the Treg percentages at baseline could affect this outcome, we stratified individuals according to Treg percentage at baseline (<10% and≥ 10%) and the CD4+ T cell counts were evaluated throughout therapy and found no differences (Figure 3A). However, at baseline, individuals with higher Treg percentages have lower CD4+ T cell counts in comparison to individuals with lower Treg percentages (211 ± 146 vs. 313 ± 243 CD4+ T cell counts, p=0.030 by unpaired T-test). For this reason, and in order to evaluate reconstitution of individuals on the same stage of lymphopenia at HAART initiation but with distinct Treg percentages, we stratified individuals first based on their CD4+ T cell counts and then on the Treg percentage, both at baseline. No differences were observed on the pathern of CD4+ T cell recovery independently of the CD4+ T cell strata at baseline (Figure 3B).

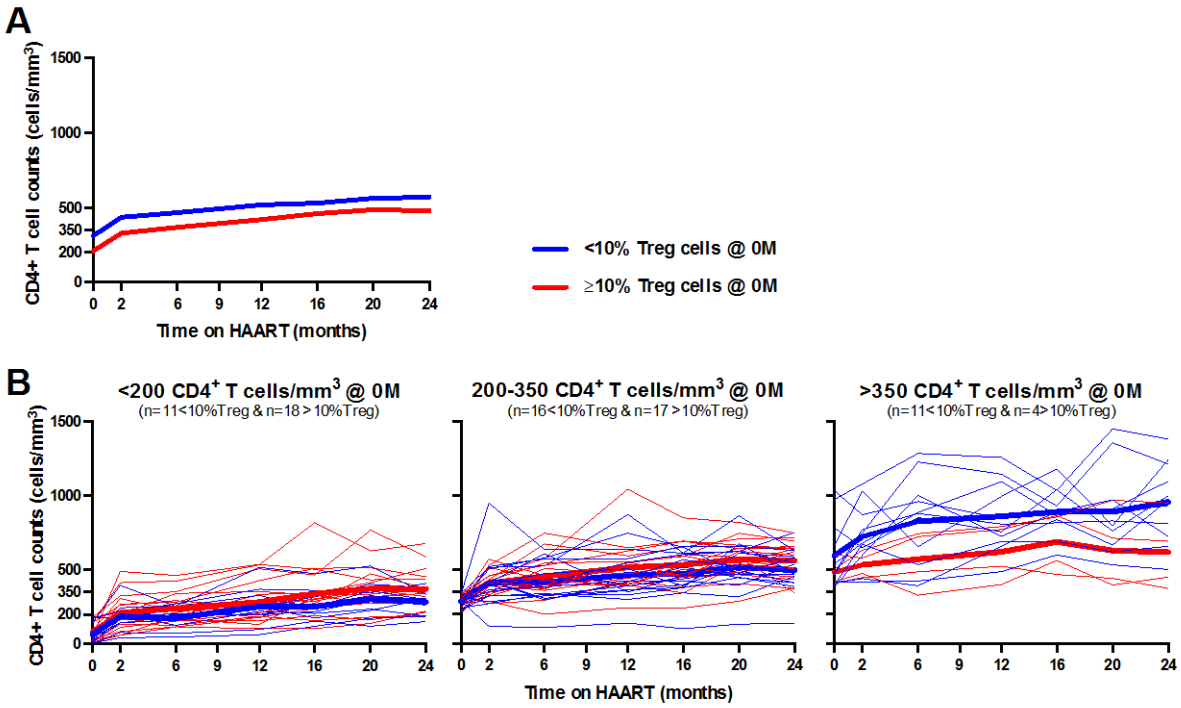

Figure 3. Evolution of CD4+ T cell counts with HAART is not associated with Treg percentage at baseline. A. Stratification of HIV-infected individuals based on their Treg percentages at baseline (<10%, n=38, blue line; ≥10%, n=39, red line) and evaluation of the CD4+ T cell reconstitution during HAART. Each line represents the median of all individuals in each specific time-point. **B.** Stratification of HIV-infected individuals based first on their CD4+ T cell counts at baseline (<200, 200-350 and >350 cells/μL) and afterwards on their Treg percentages at baseline (<10%, blue lines; ≥10%, red lines) and evaluation of the CD4+ T cell reconstitution with HAART. Each thin line represents a single individual and bold lines represent the median of all individuals from each group in each specific time-point. In

all graphs, a two-way ANOVA repeated measurements was performed to compare the evolution of the two groups throughout time of HAART and no significant differences were observed.

Taken together, this data suggest that the negative effect observed of the higher Treg percentages at baseline on the $CD4^+$ T cell reconstitution is dependent on the baseline $CD4^+$ T cell counts. To test this hypothesis, and considering that several other different factors account for the immune reconstitution (e.g. age, gender, basal viral load, residual viral replication during HAART, co-infections, immune activation status [4]), hierarchical linear regression models were performed. As can be observed in Table 2, model 1, age, gender, co-infections, viral load at baseline and at 24 months and immune activation status ($HLA\text{-}DR^+$ cells among $CD4^+$ T cells) at 24 months of HAART, by themselves are able to significantly predict 30% of the $CD4^+$ T cell counts at 24 months of HAART. When the Treg percentages at baseline were included in the model (Table2, model 2) the predictive capacity of the model increases to 35% (5% difference from the 1st model) with Treg percentages at baseline showing up as a significant predictor of $CD4^+$ T cell reconstitution. Taking all the afore mentioned variables in consideration, for the gain of each percentage unit of Treg at baseline, the predicted $CD4^+$ T cell counts at 24 months decreases in 11 cells/μL. However, when the $CD4^+$ T cell counts at baseline are taken into consideration (Table 2, model 3) the predictability of the model raises to 70% (35% and 40% difference relatively to the 1st and 2nd models, respectively) but the contribution of the Treg percentage at baseline loses its significance.

	Model 1				Model 2				Model 3			
	B	SE	β	p	B	SE	β	p	B	SE	β	p
Age[1]	-4.55	2.71	-0.18	0.10	-6.01	2.71	-0.23	**0.03**	-3.01	1.89	-0.12	0.12
Gender[2]	41.18	66.49	0.07	0.54	69.77	65.78	0.11	0.29	-31.60	46.75	-0.05	0.50
Log (HIV viral load) @ 0M	-116.58	43.97	-0.29	**0.01**	-104.25	43.02	-0.26	**0.02**	-24.57	31.01	-0.06	0.43
Chronic co-morbility[3]	-71.09	62.16	-0.12	0.26	-43.56	61.56	-0.08	0.48	-100.38	42.84	-0.18	**0.02**
Log (HIV viral load) @ 24M	-339.99	182.29	-0.19	0.07	-389.38	178.28	-0.22	**0.03**	-61.10	128.43	-0.04	0.64
HLA-DR+ among CD4+ T cells @ 24M	-6.81	1.81	-0.41	**0.00**	-6.84	1.76	-0.42	**0.00**	-2.58	1.31	-0.16	**0.05**
%Treg cells @ 0M	-	-	-	-	-10.83	4.82	-0.24	**0.03**	1.20	3.60	0.03	0.74
CD4+ T cell count @ 0M	-	-	-	-	-	-	-	-	0.91	0.11	0.73	**0.00**
F (dF1, dF2)	4.72(6.66)*				5.02(7.65)*				18.47(8.64)*			
R^2	0.30				0.35				0.70			
R^2 change	-				0.05				0.35			

Table 2. Hierarchical linear regression models to predict CD4+ T cell counts at 24 months of HAART.
[1]At baseline; [2]Reference category: female; [3]Reference category: no chronic co-morbidity (HCV infection/cancer/medical condition leading to recurrent infections; consider positive when at least one of them occurred during the 24 months of HAART). Variables were considered to significantly contribute to the model when $p<0.05$.

HIV-infected individuals showed Treg subset disturbances that are not restored with HAART initiation

The human Treg population is heterogeneous and can be subdivided in naïve and memory Treg (CD45RA$^+$ and CD45RA-, respectively), which are developmentally related cells [38, 40]. Because these cells, upon *in vitro* stimulation, present distinct phenotypes (proliferative but highly resistant to apoptosis cells vs. hyporesponsive and apoptotic cells, respectively) [38], the maintenance of the right proportion of these subpopulations is considered of relevance for the functioning of the immune system. In order to evaluate whether the Treg compartments were restored with HAART initiation to levels similar to healthy controls, we evaluated the percentage of naïve and cycling Treg.

We observed a clear disturbance in Treg subpopulations distribution; in chronically untreated HIV-infected individuals, the percentage of CD45RA$^+$ cells among total Treg was lower in comparison to healthy individuals and this difference persisted even upon 24 months of HAART (Figure 4A). These differences were more evident for individuals with less than 350 CD4$^+$ T cells/μL at baseline (Figure 4B).

As for the percentage of Treg undergoing proliferation, overall the percentage of Ki67$^+$ Treg is increased in HAART-naïve HIV-infected individuals in comparison to healthy controls though it normalizes with HAART initiation (Figure 5A). When HIV-infected individuals are stratified accordingly to their stage of lymphopenia at baseline, only the individuals with the lowest CD4$^+$ T cell counts before HAART initiation (<200 cells/μL) presented higher Ki67$^+$ cells in comparison to healthy controls. This difference is maintained up until the end of follow-up (Figure 5B).

2.3.5. Discussion

The evolution of Treg percentages during HAART and their contribution to the immune reconstitution of HIV-infected individuals is still a controversial issue that deserves attention. This lack of consensus might be related to several factors such as the type of studies performed, the heterogeneity and size of the study population, the time on therapy, the markers used to define this cell subset, among others. In an attempt to clarify this matter, we performed a longitudinal cohort study with 81 HIV-infected individuals being followed since HAART onset and for the following 24 months. During this time-period all individuals showed a decrease in their plasma

viral loads and most of them presented an increment of the $CD4^+$ T cell counts in a fashion similar to the one described previously [45]. However, it is important to notice that not all individuals are able to achieve proper immune reconstitution. Several factors have been inconsistently related with this lack of success, among them Treg.

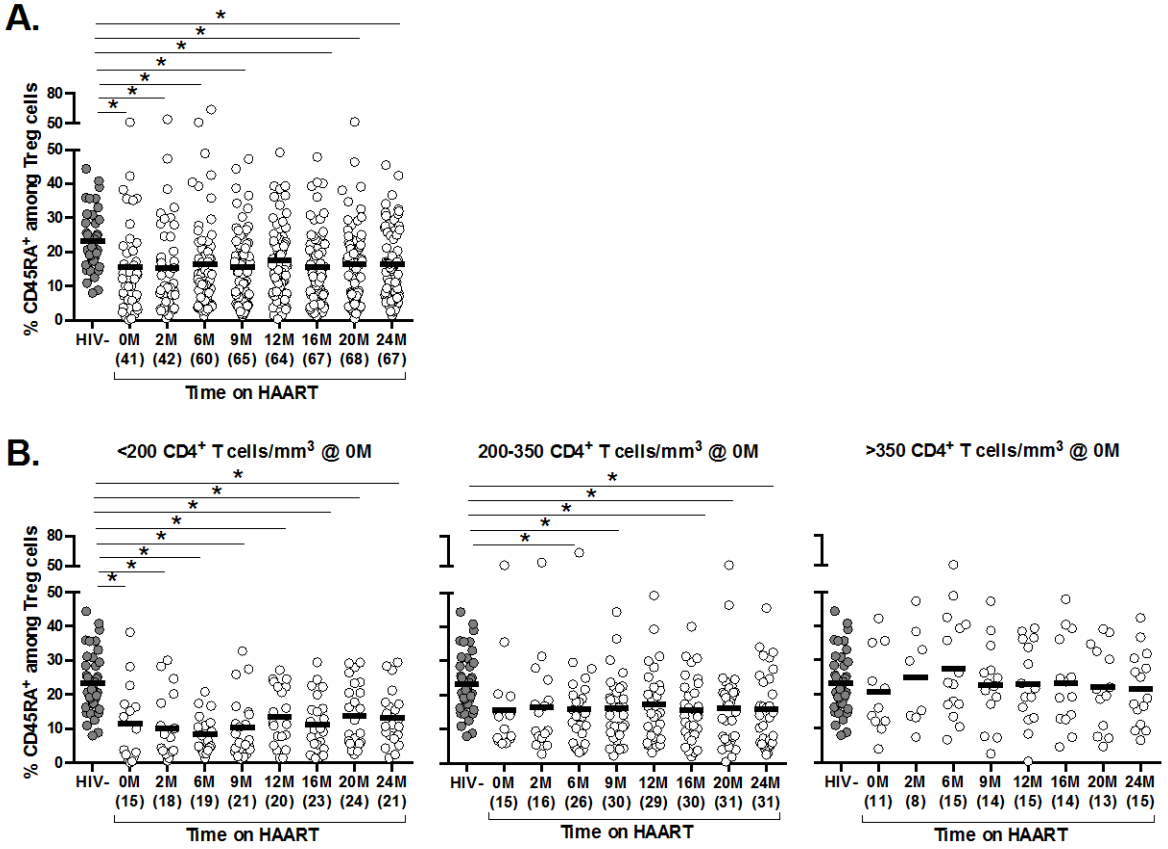

Figure 4. The percentage of naïve cells among Treg is not restored to levels similar to healthy controls at the end of 24 months of HAART. A. Percentage $CD45RA^+$ cells among Treg on healthy controls and on HIV-infected individuals from baseline up until 24 months of therapy. **B**. Percentage $CD45RA^+$ cells among Treg upon stratification of the individuals accordingly to their $CD4^+$ T cell counts at baseline. In all graphs, each dot represents a single individual; horizontal black lines represent the mean. Comparison between healthy controls and HIV-infected individuals naïve for HAART or on different time-points upon HAART initiation performed using a one-way ANOVA test followed by Bonferroni's multiple comparison tests.

As most of the studies describe [23-28], we have observed an increase of Treg percentages in HAART-naïve HIV-infected individuals in comparison to healthy controls. However, when individuals were stratified accordingly to their $CD4^+$ T cell counts at baseline this observation was more evident for individuals with less than 200 $CD4^+$ T cells/μL at baseline, indicating that Treg evolution with HAART varies accordingly to the state of immunosuppression at the beginning of therapy. In fact, the lack of alterations in the Treg percentage of HIV-infected

individuals with CD4+ T cell counts above 200 cells/μL goes along with the observation made by Simmoneta *et al.* that evaluated a cohort of individuals with CD4+ T cell counts at baseline above 350 cells/μL [14]. Thus, we added to the established knowledge that, although in general the percentage of Treg is increased in the HAART-naïve HIV-chronically infected individuals, the diversity is very high and the higher Treg percentages are clearly enriched among the individuals with very low CD4+ T cells.

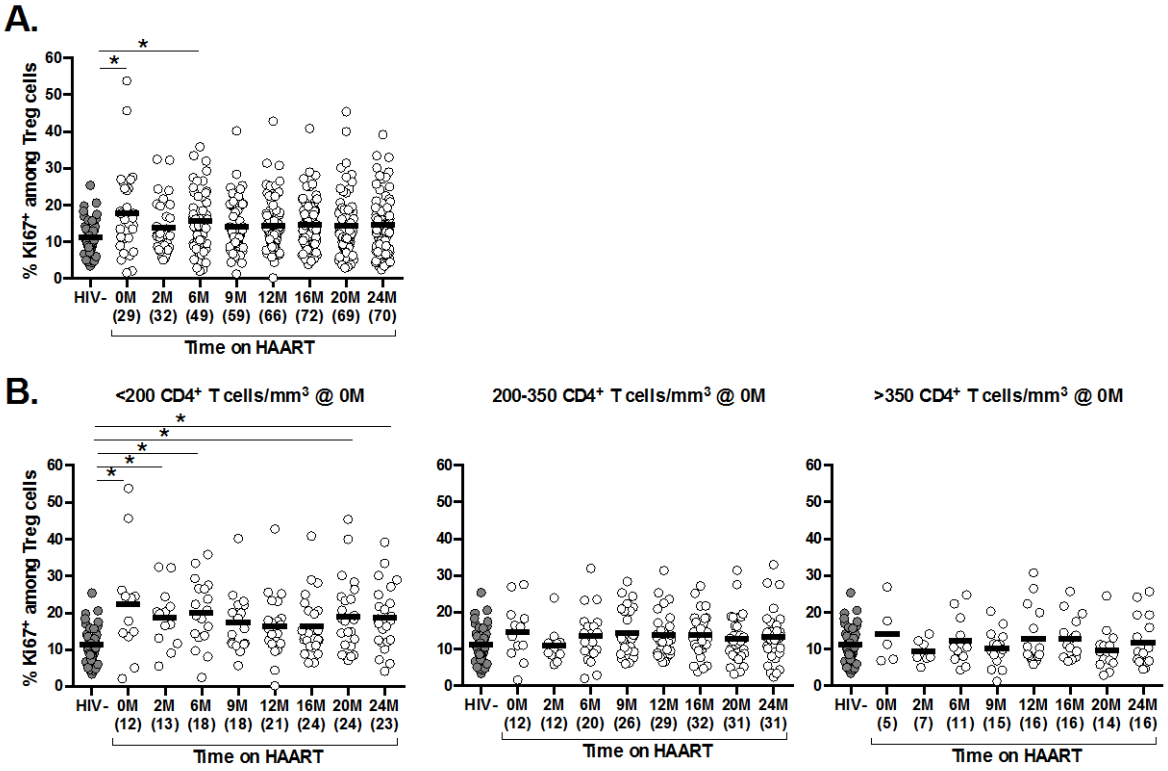

Figure 5. Highly lymphopenic individuals at baseline maintain high proportions of Treg undergoing proliferation throughout HAART. A. Percentage Ki67+ cells among Treg on healthy controls and on HIV-infected individuals from baseline up until 24 months of therapy. **B**. Percentage Ki67+ cells among Treg upon stratification of the individuals accordingly to their CD4+ T cell counts at baseline. In all graphs, each dot represents a single individual; horizontal black lines represent the mean. Comparison between healthy controls and HIV-infected individuals naïve for HAART or on different time-points upon HAART initiation performed using a one-way ANOVA test followed by Bonferroni's multiple comparison tests.

Disturbances in Treg subsets homeostasis in chronically untreated HIV-infected patients have been described in other studies [14, 32, 34, 46-48]. Some of these reports evaluated the percentages of Treg subsets among CD4+ T cells [14, 47]. However, because the percentage of total Treg among CD4+ T cells is highly variable for HIV-infected individuals (being or not on HAART), looking at percentages of Treg subsets among total CD4+ T cells, and not among Treg cells specifically was not the best option to analyse the diversity between patients. So, taking into

account only studies that evaluate naïve cells among Treg, the study by Gaardbo *et al.* observed that the percentage of naïve cells in healthy controls is higher than that in HIV-infected individuals on HAART, irrespective of their outcome in what concerns immune reconstitution (non-responders, intermediate responders and responders) [48]. On the other hand, on the study published by Serana *et al.* no significant differences were observed on the percentages of this Treg subset in healthy controls vs. individuals on long-term HAART (>6 years) [46]. The discrepancy on these results might now be justified in light of our data as we observe that the evolution of the naïve cells among Treg varies accordingly to the $CD4^+$ T cell counts at baseline. Nevertheless, the low percentages of naïve cells among Treg may reflect an increased turnover of these cells into memory phenothype. In fact, we observe higher percentages of Treg cells undergoing cell division for the most immunosuppressed individuals at baseline (<200 cells/μL), even after initiating HAART, in comparison to the healthy controls. However, as natural Treg are generated in the thymus [38], one may not exclude the possibility that lower percentages of naïve cells among Treg could be related to lower thymic output, since it is known that HIV infection impacts thymic function [49].

Several cross-sectional reports have proposed a role for Treg on the accomplishment of immune reconstitution [18, 31-34, 50]. In fact, we have previously shown that individuals with impaired immune reconstitution (i.e. less than 500 $CD4^+$ T cells/μL after 1 to 15 years on HAART) were the ones with the highest Treg percentages (≥10%) and lower nadir values (the lowest $CD4^+$ T cell count ever achieved by an HIV-infected individual) [33]. More recently, Saison *et al.*, observed in a multivariate model adjusted for age, nadir, Treg percentage and $CD8^+$ T cell activation ($CD38^+$) that nadir and Treg percentages were the only two parameters associated with immunological response to HAART [31]. To our knowledge, no reports until now are available on the impact of Treg percentages at baseline on the immune reconstitution process. In our point of view this is a relevant issue especially if we take into consideration the observed high variability of Treg percentages at baseline. In fact, we perceive that Treg percentages at baseline correlates negatively with $CD4^+$ T cell counts at 24 months of HAART. However no differences are observed on the $CD4^+$ T cell counts progression of individuals with high vs. low Treg percentages at baseline (<10% and ≥10%) even after being stratified accordingly to their baseline $CD4^+$ T cell counts. Taking into account in hierarchical linear regression models other factors known to affect immune reconstitution we observed that Treg percentages at baseline affect $CD4^+$ T cell counts at 24 months in a way dependent of $CD4^+$ T cell counts at baseline. Despite the fact that some

reports show no alterations on the suppressive capacity of Treg during HIV-infection [51], recently, the reports by Pion *et al.* [52] and by Angin *et al.* [53], have described *in vitro* that HIV-infected Treg show impaired suppressive capacity and a decreased expression of genes critical to Treg function. We have no information on the suppressive function of the Treg cells from our patients which is a weakness of the present study. We observed that Treg percentages at baseline positively correlate with the percentages of $CD4^+$ T cells with an activation phenotype ($HLA\text{-}DR^+$) and undergoing proliferation ($Ki67^+$; data not shown). *In vitro* studies should be performed to evaluate Treg suppressive capacity from individuals with the highest Treg percentages among the ones with less that 200 $CD4^+$ T cells/μL.

Taken together, our data shows that the evolution of Treg proportions and of its subpopulations in the onset of HAART varies differently accordingly to the baseline state of immunosuppression. Moreover, we show for individuals with very low $CD4^+$ T cell counts at baseline that the Treg percentage is extremely diverse which might explain some of the controversy on the data previously published by others. In addition we show that the Treg cell subsets are disrupted due to lymphopenia and the normal proportion of these Treg cell subsets is not recovered, even when the $CD4^+$ T cell counts rise to numbers similar to non-infected individuals. Our work adds to others that very low nadir leads to disruptions at different levels of the T cells and that part of these alterations are not recovered during HAART. Further studies should be carried out to evaluate the suppressive capacity of Treg in HIV-infected individuals with very low numbers of $CD4^+$ to determine if the great diversity in percentage is associated with diversity also in function. Finally, these patients, as well as other cohorts, have to be followed for longer periods to clearly define the role of Treg, and other cells on the quality of the immune reconstitution of HIV-infected patients under HAART.

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2.4. Newly Detected Spinal Cord Lesions in a Patient Infected with HIV, with a History of Cerebral Toxoplasmosis under Correct Treatment – a Case of Immune Reconstitution Inflammatory Syndrome and regulatory T cells deregulation?

A Case Report

Ana Horta[1,2,3,*], Claudia Nobrega[1,2,*], Eugénia Reiriz[3], Soraia Almeida[3], Miguel Abreu[3], Rui Sarmento-Castro[3], Margarida Correia-Neves[1,2]

1 Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal.

2 ICVS/3B's, PT Government Associate Laboratory, Braga/Guimarães, Portugal.

3 Infectious Diseases Service of the Centro Hospitalar do Porto, Hospital Joaquim Urbano Unit, Porto, Portugal.

* These authors contributed equally to this paper

2.4 Newly Detected Spinal Cord Lesions in a Patient Infected with HIV, with a History of Cerebral Toxoplasmosis under Correct Treatment – a Case of Immune Reconstitution Inflammatory Syndrome and regulatory T cells deregulation?

A Case report

2.4.1. Abstract

Background: Patients infected with HIV, especially in advanced stages of AIDS, are frequently affected by neurological disorders. Neurological disorders in AIDS patients might be a consequence of several mechanisms like: direct HIV infection of cells from the central nervous system (CNS); infection of the central nervous system by opportunistic pathogens; or immune reconstitution inflammatory syndrome (IRIS). Despite the great prevalence of toxoplasmic encephalitis, only few cases of neuro-IRIS related to this AIDS defining condition have been described.

Case presentation: We present a case of a patient infected with HIV that upon being successfully treated for a cerebral toxoplasmosis unexpectedly deteriorated two months after antiretroviral therapy initiation and developed lesions in the spinal cord. Since this patient was included in our longitudinal cohort, it was possible to follow the values of regulatory $CD4^+$ T cells (Treg) and Treg subsets from the moment of antiretroviral treatment onset and throughout all events that occurred thereafter. We have also been able to compare the results obtained with those presented by nine matched patients also from the longitudinal cohort as controls.

Conclusions: The case presented is strongly suggestive of a paradoxical toxoplasmosis-IRIS with the involvement of the spinal cord. The rarity, not only of the toxoplasmosis related IRIS, but also the localization of the lesions (spinal cord), renders this presentation a case of particular interest. In what concerns Treg and Treg subsets, although we have found a large diversity of the values and in their evolution over time after antiretroviral treatment initiation between all patients (case and controls), the variation of those values in the case seems to be more pronounced than in controls, and more pronounced in the first three months of antiretroviral therapy coinciding with the manifestations of IRIS.

Keywords: CNS inflammation, HIV/AIDS, IRIS, Toxoplasmosis, myelophaty

2.4.2. Introduction

The vast majority of untreated HIV-infected patients suffer from a gradual decrease of the $CD4^+$ T cell counts rendering patients highly susceptible to several opportunistic diseases that can occur in almost all the body systems including the central nervous system (CNS). Agents that are known to be involved in neurological complications in AIDS patients are on one hand the HIV itself [1] and on the other hand opportunistic pathogens [2]. Furthermore, in patients initiating HAART and within the first weeks or months, the recovery of the $CD4^+$ T cells might be followed by an aberrant pathogen-specific immune response, leading to a heightened inflammatory process and, consequently, to a worsening of the state of health of the patient. This process has been defined as immune reconstitution inflammatory syndrome (IRIS). IRIS occurs following the restoration of the host defenses against an active living and replicating pathogen (previously subclinical and undiagnosed opportunistic infection – unmasking IRIS) or against a non-active residual pathogen/antigens (opportunistic infection previously diagnosed and correctly treated – paradoxical IRIS) [3].

IRIS was first reported in 1992 [4]. Since then, it became a distinct clinical condition affecting a growing number of patients due to the increasing use of HAART worldwide [5]. The frequency of IRIS among HIV-infected patients starting HAART, in a meta-analysis involving 54 cohort studies from 22 countries (of high, high-middle, low-middle, and low income), was estimated at 16% (11,1 – 22,9%) with 4,5% (2,1 – 8,6%) mortality [6]. IRIS is a challenging condition as the clinical features are nonspecific and consequently, there is no consensual case definition. Since the immunopathogenesis of IRIS is poorly understood, the optimal preventive and treatment strategies are still controversial [7].

Several immune events occur during immune restoration that might, independently or synergistically, lead to IRIS [7 and references therein]. Data from numerous reports suggest deregulated $CD4^+$ T and/or natural killer (NK) cell responses as responsible for the exacerbated inflammation response and consequent tissue damage [7 and references therein].

During IRIS, a powerful and aberrant pathogen-specific immune response by conventional T cells occurs, and some events concerning these cells could in part explain this exacerbated antigen-specific immune reaction in a IRIS scenario: 1) due to the previous encounter between immune cells and specific antigens of the pathogen, occurred an expansion of memory T cell ($CD4^+$ and $CD8^+$ T cells residing in lymphoid tissues) which specificities are

limited in diversity [8]; 2) this limited diversity might be even enhanced in lymphopenic scenarios as these situations lead to a homeostatic proliferation of T cells that are T cell receptor (TCR) stimulation dependent [9]; 3) moreover, these latter memory T cells, expanded during proliferation lymphopenia-induced are more permissive to commit into effector T cells as a result of the activation by present antigens [10]; 4) since that the first rapidly peripheral increase in $CD4^+$ T cells after HAART initiation is caused by a redistribution of memory $CD4^+$ and $CD8^+$ T cells from lymphoid tissues to the periphery [11], the highly specific and asymmetrical expanded memory T cells already resided within tissues reach the periphery [12]; and also, 5) a potential contribution to be considered is an autoimmune response - self-antigens derived from debris and/or from the uptake of dying cells by antigen presenting cells could turn T and B cell responses able to perpetuate tissue damage [13].

The regulatory T cells (Treg), a subset of $CD4^+$ T cells, are essential to blunt the immune-response, helping to maintain the immune system homeostasis and protecting the host from exaggerated immune-mediated damage. These cells have also been suggested to be involved in IRIS deregulated immune response by several reports [7,14-17]. It is known that after HAART initiation, a rapid rebound of conventional T cells is not always followed by a parallel raise of Treg which could lead to a decreased proportion of Treg in respect to the conventional $CD4^+$ T cells [14]. However, Treg proportion has also been demonstrated to raise in patients suffering from IRIS suggesting that although present, Treg might be dysfunctional [15,16,17], or that the new conventional $CD4^+$ T cells might be refractory to Treg function [14].

IRIS affecting the CNS (CNS-IRIS) presents, compared with other IRIS-associated organ, the aggravating circumstance that the CNS is of poor access to clinical investigation and that CNS-IRIS is associated with greater morbidity and mortality, frequently resulting in severe neurologic disability or even death [3]. CNS-IRIS is estimated to occur in 0.9 to 1.5% of patients during the first months on HAART [18]. Despite the fact that toxoplasmic encephalitis is the most prevalent opportunistic infection of the CNS among AIDS patients, only few cases of unmasking and, even less of paradoxical toxoplasmic encephalitis-IRIS have been reported [7,19-24]. In addition, the involvement of the spinal cord in AIDS associated toxoplasmosis is rare [19,25,26], and only one case report of paradoxical toxoplasmic medullar-IRIS has been published [19]. The strong ability of *Toxoplasma gondii* to evade the immune system (with mechanisms to reduce its visibility to the immune system by decreasing the expression of immunogenic surface proteins and maintaining a low-metabolism), and to hamper an immune response (favoring the expansion

of Treg and interfering with the MHC class I and class II antigen presentation pathway and interferon-γ signaling) could explain the low predisposition of this pathogen to cause CNS-IRIS [7,19,20,27,28]. Hence we consider important to present here a strongly suggestive case of paradoxical toxoplasmosis IRIS with spinal cord involvement. Moreover it was interesting to investigate the dynamic of Treg and its subsets in parallel with all events occurred after HAART onset.

2.4.3. Case presentation

Four months after HIV diagnosis and three months after HAART onset, a 37-year-old man presented (Figure 1), in a routine medical appointment, decreased sensory sensation of the right lower limb that lasted for about 1 month. The beginning of the hyposensitivity had been somewhat abrupt but progressively worsening. At the moment of the medical appointment, it extended to the entire right lower limb and half of the abdomen. He showed no fever, headaches, vomiting, vertigo, numbness, weakness, seizures, other neurologic, and neither symptom of the respiratory, digestive, or urinary systems.

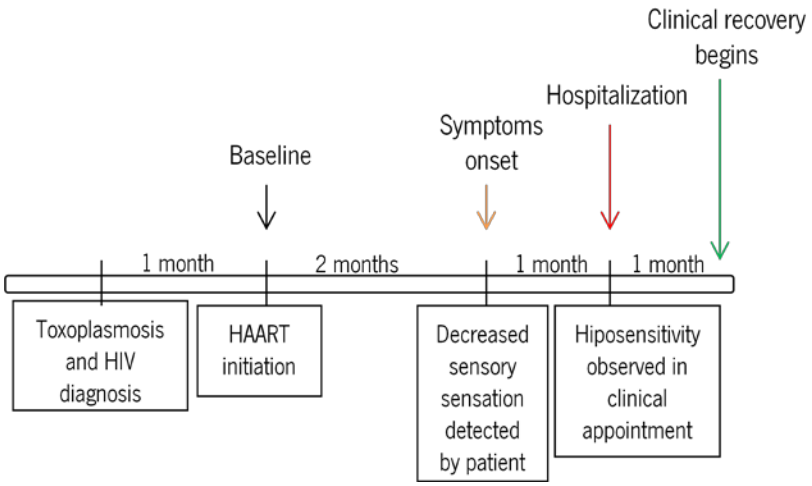

Figure 1. Events chronology. The color arrows point out crucial events and will be used in the Figures 8-12.

When HIV infection was diagnosed (four months before), the patient presented headache, psychomotor retardation and left hemiparesis, and a diagnosis of cerebral toxoplasmosis was made, based on: diagnosis of HIV infection, advanced immunodeficiency ($CD4^+$ T cell count of 20/mm^3, and a plasma HIV-1 load of 301000 copies/ml), a computed tomography scan (CT) showing scattered lesions in the brain parenchyma with perilesional

edema, mass effect, and some with ring contrast enhancement (Figure 2), a positive serology for toxoplasmosis, and a clinical and radiologic improvement after specific anti-toxoplasmosis treatment without corticosteroid therapy (a CT scan, performed 14 days after the initiation of the anti-toxoplasmose theraphy showed scattered smaller lesions with only little residual edema and no ring contrast enhancement – Figure 3).

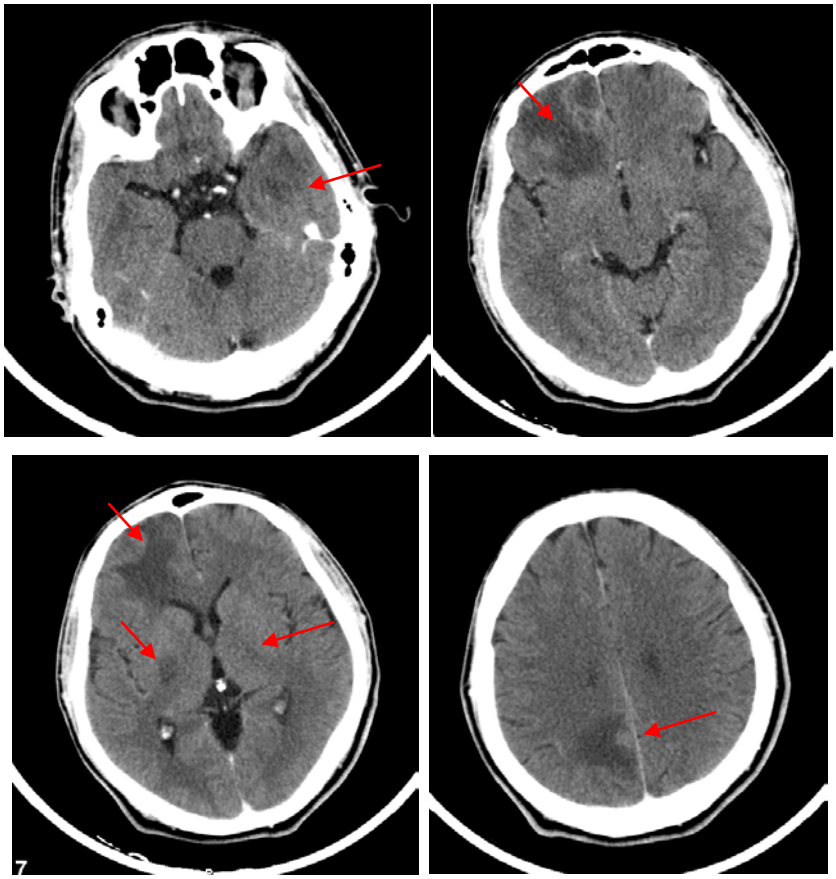

Figure 2. First brain CT, after contrast injection. Multiple hypo-density lesions, perilesional edema and sulcal effacement suggesting mass effect. After contrast injection, some of these lesions revealed ring enhancement.

HAART (with tenofovir, emtricitabine and darunavir boosted with ritonavir) was initiated one month after HIV diagnosis, toxoplasmosis diagnosis, and specific anti-toxoplasmosis treatment onset (Figure 1). Toxoplasmosis treatment was maintained during two and an half months and then changed to suppressive therapy whereas prophylaxis for other primary opportunistic infections was maintained.

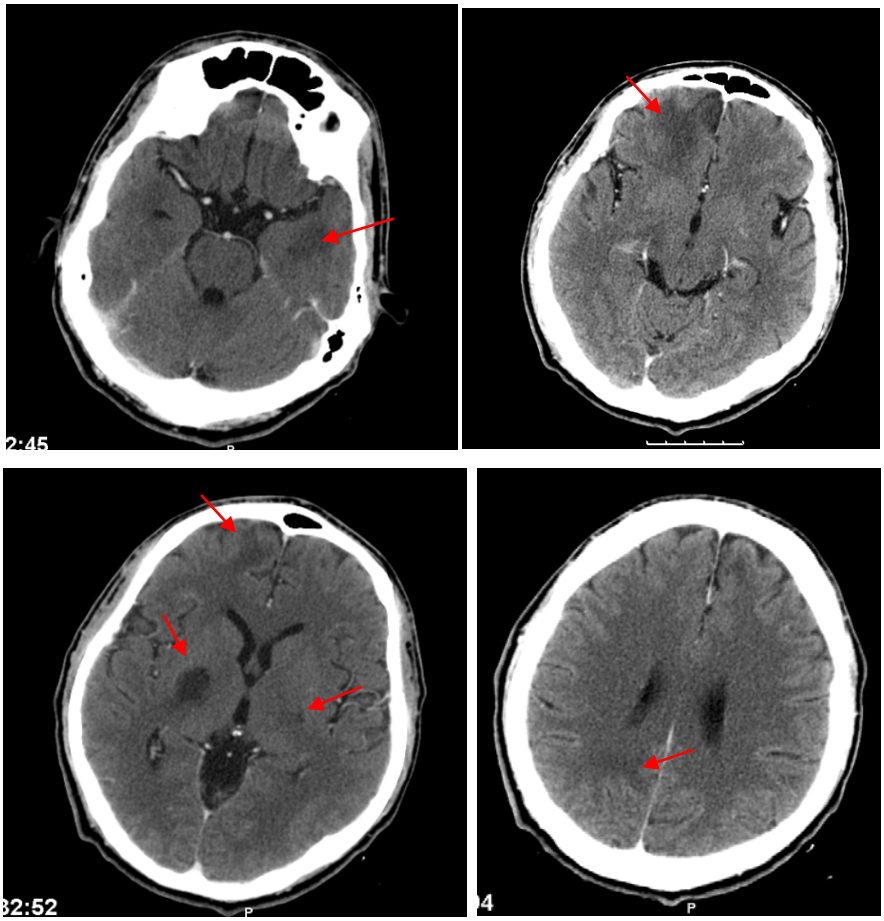

Figure 3. Brain CT realized after 14 days under specific anti-toxoplasma therapy, after contrast injection. Improvement of previous lesions is seen. No more mass effect, or ring contrast enhancement were seen.

Three months after HAART initiation, in the above mentioned routine medical appointment, the patient stated that he was always taking his medication correctly: HAART, suppressive therapy for toxoplasmosis, and primary prophylaxis for *Mycobacterium avium complex* disease. Despite a slight sequelar left hemiparesis, the mental status of the patient was normal, no papilledema or neck stiffness were present, and the cranial nerves exam was normal, but an upper right unilateral sensory level at D-9 was present with loss of touch sensation and of the ability to feel pain bellow this level across the entire right lower limb and right hemi-abdomen. The position sensation was normal, and normal muscular force and reflexes were present in the correspondent area. The patient was immediately admitted to the hospital to be studied:

- A cerebral CT scan was performed (Figure 4) revealing the previous lesions (a couple revealing worsening with a slight ring contrast enhancement and perilesional edema, others revealing improvement), and some new lesions. A magnetic resonance imaging (MRI) of the dorsal spinal cord (Figure 5) revealed myelitis: multiple areas of high signal intensity on the T2-weighted images (one of them, at D4, presenting contrast enhancement on T1-weighted images),

located between D3 and D9, mainly peripheral and on the left side, and probably a posterolateral lesion on the right side at D7-D8.

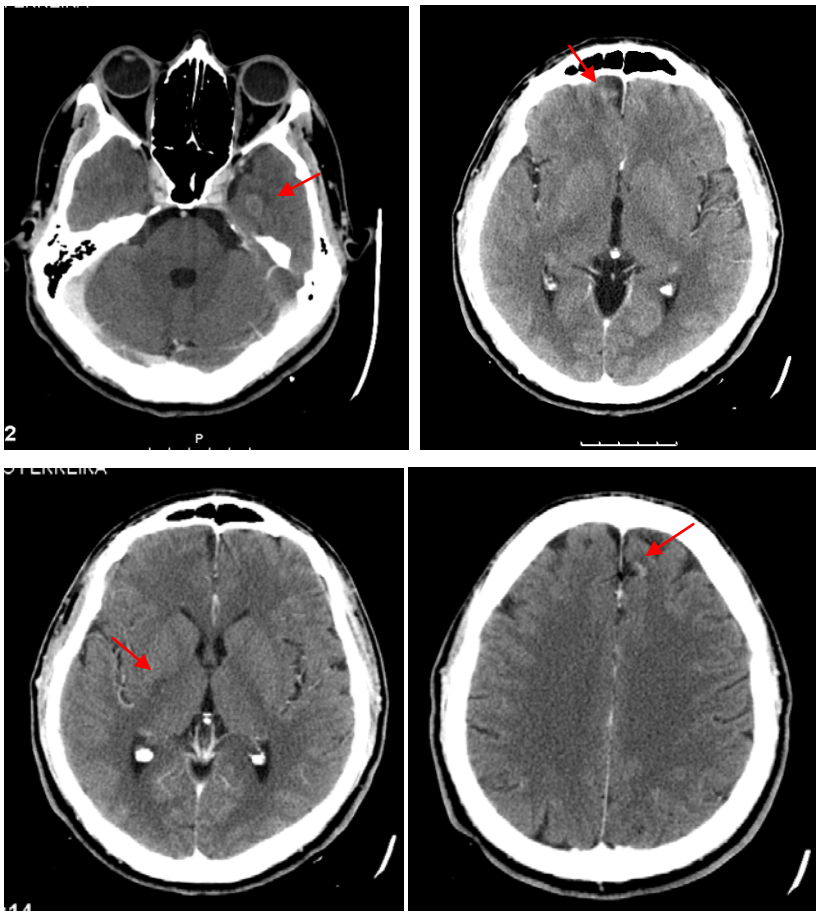

Figure 4. Brain CT after contrast injection. Several hypo-density lesions, some of them with slight perilesional edema and ring enhancement after contrast enhancement.

- The $CD4^+$ T cell count was 128/mm^3 and the plasma HIV-1 viral load, 121 copies/ml. The cerebrospinal fluid (CSF) evaluation revealed the following values: 5 cells/ml; 58 mg/dl of protein; 51 mg/dl of glucose; negative results for stain and cultures for bacteria (Gram and acid-fast) and fungi; negative results for the cryptococcal antigen; a nonreactive Venereal Disease Research Laboratory (VDRL) test; and the results from the polymerase chain reactions (PCR) to identify other common agents (JC virus, BK virus, cytomegalovirus, herpes simplex virus, human herpesviruses 6, varicella-zoster virus, and enterovirus) were also negative. A CSF PCR for *Toxoplasma gondii* was not performed due to technical limitations. The CSF PCR for Epstein-Barr virus was positive; but a normal CSF lymphocyte phenotype assay and a whole-body positron emission tomography scan (PET) revealing neither hyper metabolic cerebral nor medullar lesions, ruled out a CNS lymphoma. Serological HTLV-1/II antibody assay was also negative.

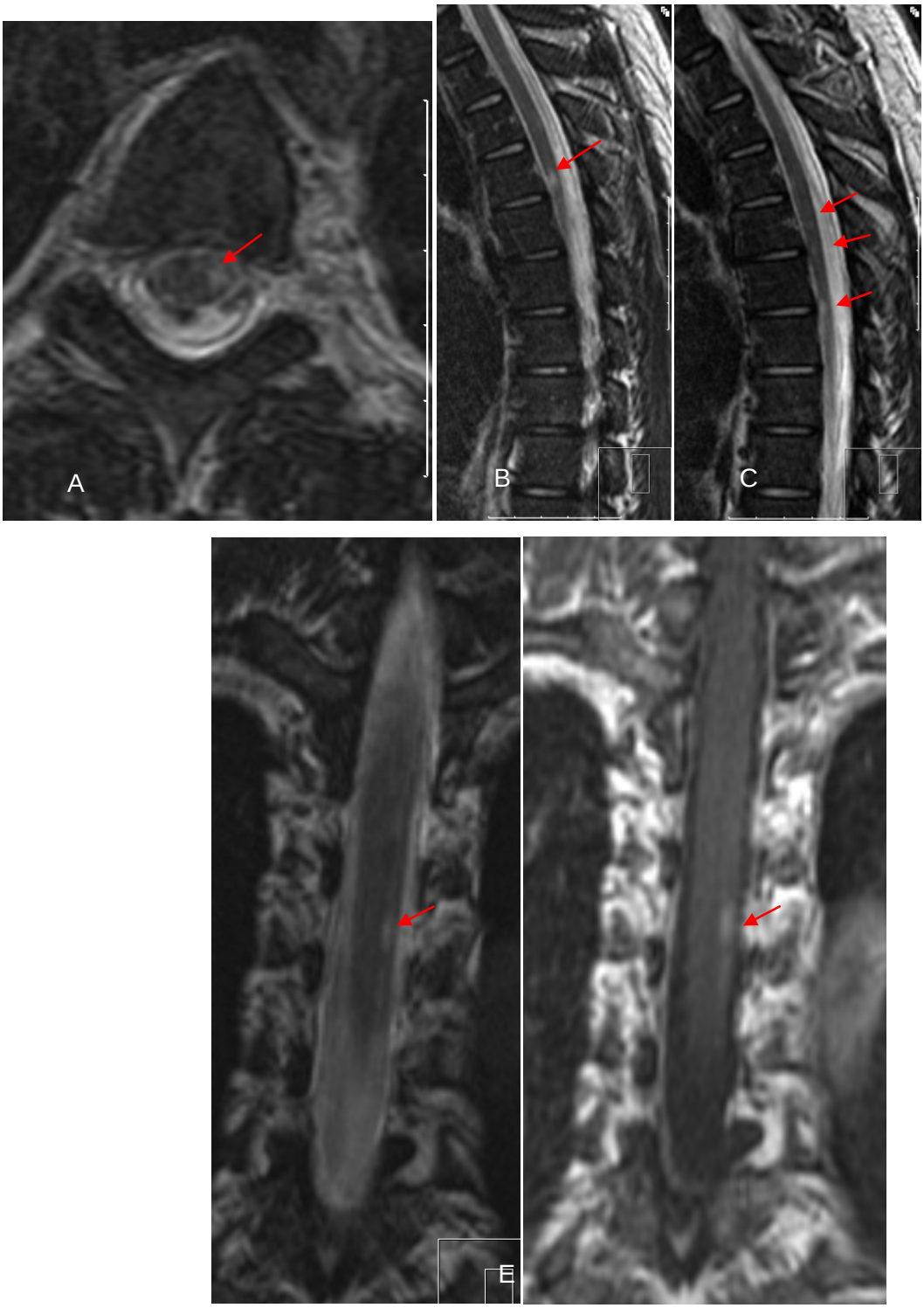

Figure 5. Spinal cord MRI **A and B.** Area of high signal intensity on the T2-weighted images, peripheral and on the left side on D4. Axial and sagittal planes respectively. **C.** Areas of high signal intensity on the T2-weighted images (sagittal plane), below D4. **D.** Area presenting contrast enhancement on T1-weighted images on coronal plane (D4). **E.** Area of high signal intensity on the T2-weighted images on coronal plane (D4).

Although the clinical and laboratory information suggested a potential case of CNS-IRIS, the toxoplasmosis suppressive treatment was changed to initial treatment by precaution, and HAART and prophylaxis for opportunistic infections maintained. Corticosteroid therapy was not

administered. One month after the anti-toxoplasmosis treatment re-initiation, the clinical status of the patient was stable, and indeed, a new brain and medullar MRI performed was similar to that performed one month before (images not shown). A stereotactic brain or medulla biopsy was considered of relevance. However, while we were waiting for the possibility to perform this intervention, the patient showed a slight spontaneous improvement of the symptoms, the biopsy was delayed and a new brain and medulla MRI was schedule to four months later (the patient was maintained under surveillance). In fact, the patient steadily improved his clinical status confirmed by the brain and medullar MRI performed. That MRI revealed the disappearance of some previous lesions and the improvement of the others (one of them now with clastic aspect – image not shown) in the brain and the complete resolution of the lesions in the medulla (Figure 6). The patient presented then fully sensory recovery of the right lower limb, only maintaining the sequelar left hemiparesis. The $CD4^+$ T cell count was 244/mm^3 and HIV viral load below the detection limit (20 copies/ml). He maintained HAART and suppressive treatment for toxoplasmosis.

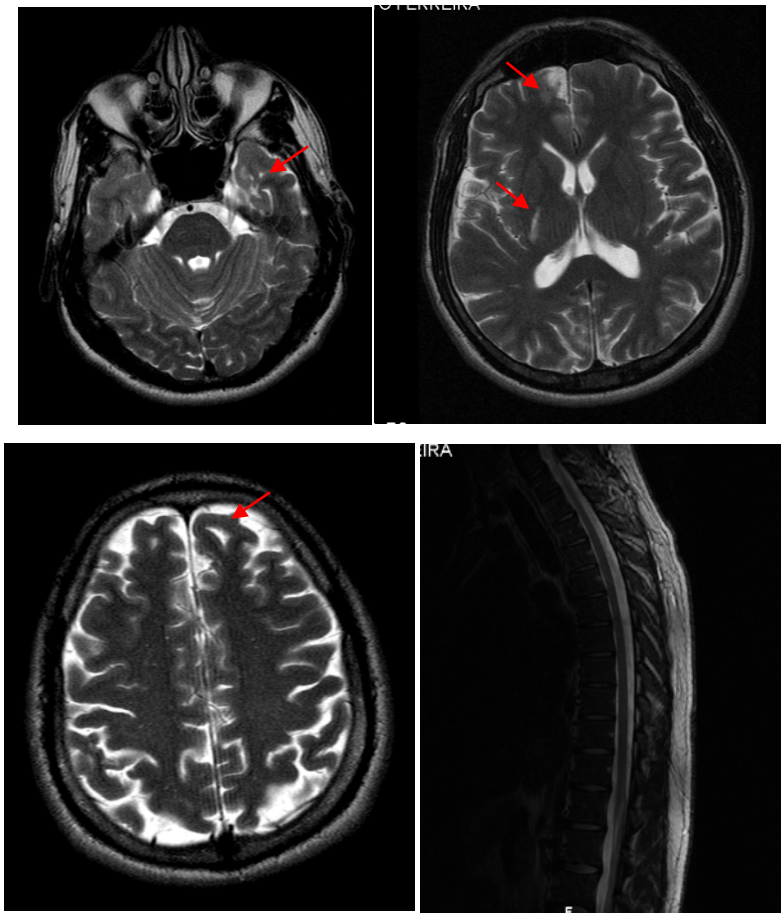

Figure 6. Brain and medullar (T2-weighted images) MRI **A:** Improvement of previous brain lesions **B:** Absence of spinal cord lesions.

The evaluation of Treg and its subsets percentages was performed over time on HAART and analyzed in parallel with the clinical course of the case, and in other nine patients as controls. The nine controls were individuals also from our longitudinal cohort (subchapter 2.1) with similar characteristics (gender, age, $CD4^+$ T cell count at baseline) as the individual of the clinical case (Figure 7), but for which neither opportunistic infections were diagnosed at baseline neither the control-patients developed signs of IRIS.

	Clinical case	Control group (n=9)
Gender Male/Female	Male	6/3
Age, years Media (range)	37	39,7 (27-48)
$CD4^+$ T cells at baseline cells/mm^3 Median (range)	20	46 (8-97)

Figure 7. Demographic characterization of the clinical case and control group

HAART resulted in a rapid decline of the plasma viral load and in an improvement of $CD4^+$ T cells count both in the clinical case and controls (Figure 8). Both the decrease in HIV viral load and the increase in $CD4^+$ T cells of the case seemed to be similar to those of controls.

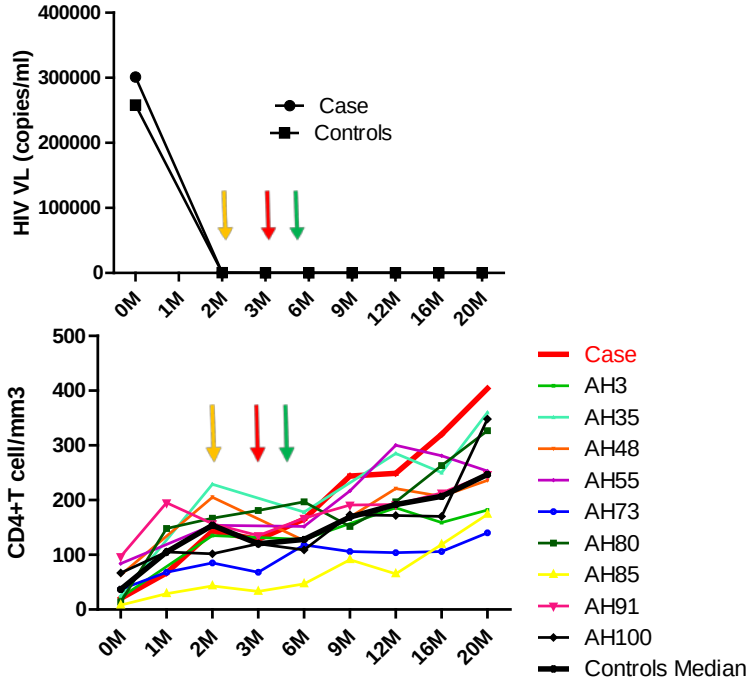

Figure 8. Decrease in HIV viral load and increase in $CD4^+$ T cells under HAART in clinical case and controls. The color arrows point out crucial events in the clinical case and are defined in the Figure 1.

Concerning the total Treg percentage on total CD4+ T cells (Figure 9), we noted in the clinical case, a decline in the first month of HAART immediately followed by a pointy rise, peaking at third month of HAART. This peak was coincident with the peak of symptoms of IRIS. After the third month, the Treg percentages progressively went back to the value observed before IRIS symptoms manifestation coinciding with clinical recovery. From 6 months on, Treg percentages of the case did not greatly differ from the majority of the individuals in the control group (Figure 9).

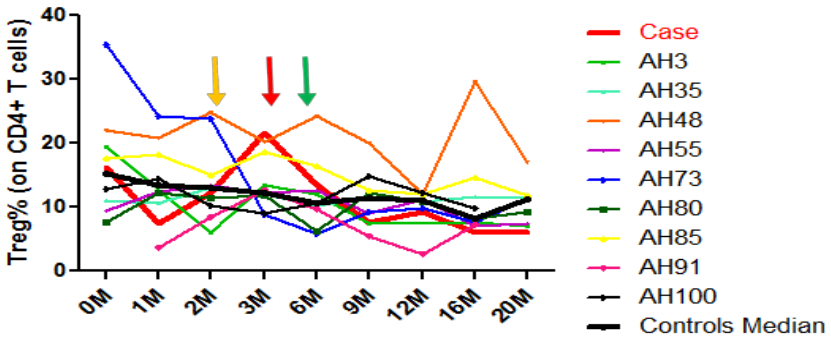

Figure 9. Total Treg frequency on total CD4+ T cells, we noted a sharp decline in the first month of HAART immediately followed by a pointy rise, peaking at third month. The color arrows point out crucial events in the clinical case and are defined in the Figure 1.

As Treg can be separated into functionally and phenotypically different subpopulations [33] we next, analyzed the Treg subsets (Figure 10) over time. And we also analyzed the dynamic of percentages of cycling Treg (Ki67+) and of recent thymus emigrants Treg (using the markers CD45RA+CD31+) among Treg [34].

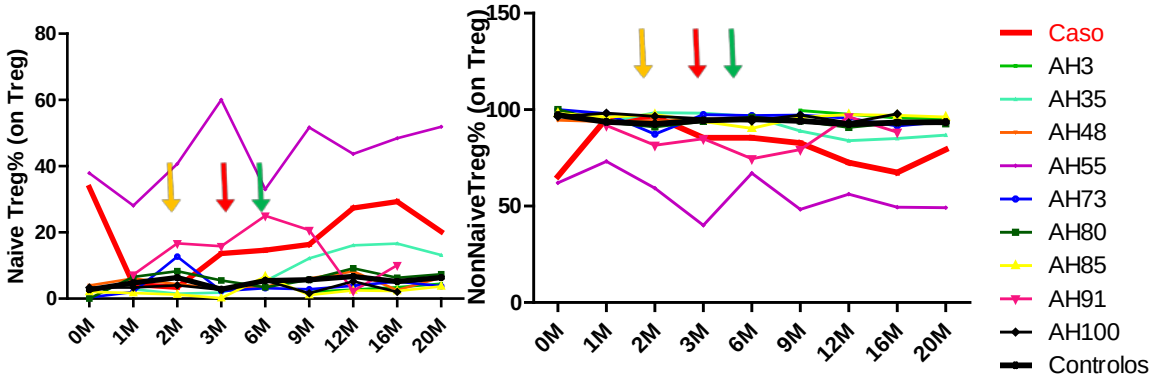

Figure 10. The naïve Treg (CD45RA+ on Treg) and non naïve Treg (CD45RA- on Treg), percentage on Treg, dynamic over time. The color arrows point out crucial events in the clinical case and are defined in the Figure 1.

We found that the first decrease, in the first month, in Treg percentage was synchronized with a sharply decrease in naïve Treg percentage (CD45RA+ among Treg) and, as it will be

expectable, with an increase on memory Treg percentage (CD45RA$^-$ on Treg) (Figure 10). We also found an increase in the percentage of cycling Treg and a decrease in percentage of recent thymus emigrants Treg (Figure 11). The high proliferation rate of Treg could have augmented the conversion rate of naïve Treg in memory Treg [33].

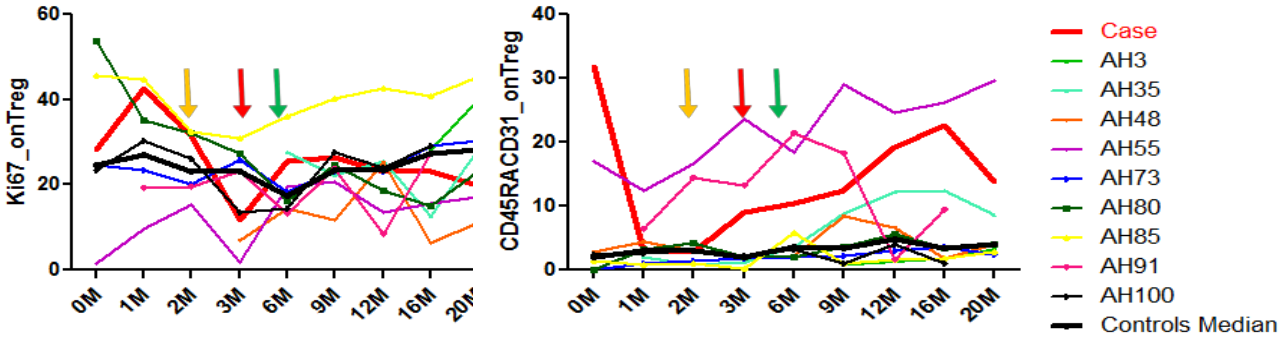

Figure 11. The dynamic of percentages of cycling Treg (Ki67$^+$) and recent thymus emigrants Treg (CD45RA$^-$CD31$^-$), on Treg, over time. The color arrows point out crucial events in the clinical case and are defined in the Figure 1.

During the second month (1 to 2 months of HAART), we noted that almost all Treg in clinical case seemed to be non naïve Treg (activated plus non suppressive Treg) (Figure 10), the percentage of cycling Treg decreased and that the percentage of recent thymus emigrants Treg was maintained at a very low level (Figure 11). Still, there was an increase in the Treg percentage (on total CD4$^+$ T cells). It is worth to note that IRIS-related symptoms initiated at the end of the second month when practically all Treg were non-naïve Treg (Figure 10).

After the second month and until the third month on HAART, when the symptoms peaked, we noted a progressive increase in naïve Treg over a decrease in non naïve Treg, that the percentage of cycling Treg continued to decrease and that the percentage of recent thymus emigrants Treg begun to increase.

After the third month on HAART, the Treg and Treg subsets changes were less evident showing a tendency to stabilize. Yet it is worth noting the sustained increase verified on percentage of recent thymus emigrants Treg and percentage of naïve Treg (Figures 10 and 11).

It is also worth to note that in the reported case, the percentage of cycling CD4$^+$ T cells (Ki67$^+$ on CD4$^+$ T cells) (Figure 12) showed the highest peak comparing to controls. That peak was noted at the end of the first month, one month before the symptoms appearance.

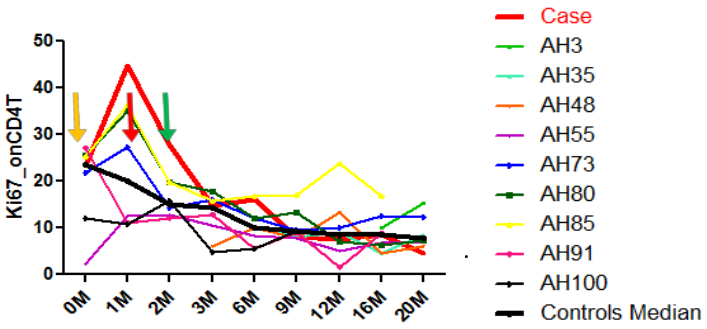

Figure 12. Ki67$^+$ on CD4$^+$ T cells (%) over time. The color arrows point out crucial events in the clinical case and are defined in the Figure 1.

2.4.4. Discussion

Although no firm conclusions can be drawn, we are strongly convinced this patient presented a case of paradoxical toxoplasmosis-IRIS with the involvement of the spinal cord.

The initial event was clearly due to toxoplasmic encephalitis – the high incidence of this disease among immunecompromised HIV-patients, the presence of typical brain lesions, the positivity of toxoplasma serology, and the improvement under specific anti-toxoplasma therapy, strongly support our diagnosis. We did not use corticosteroid therapy that could cause the false feeling of improvement as its use would decrease brain swelling whether or not the mass was caused by infection. Although CSF PCR for *T. gondii* was not performed, its negativity would not exclude the diagnosis because the sensitivity of the exam is only 50% [29].

The diagnosis of a CNS-IRIS, since there are no specific biomarkers, is presently made on the basis of a multi-parametric assessment [7] that was identified in this patient, namely: 1) a high pathogen load as revealed by the multiple brain lesions; 2) a positive response to HAART with evidence of controlled HIV replication (the HIV load decreased from 301000 to 121 copies/ml in 3 months) and a immunological improvement (the CD4$^+$ T cell count increased from 20 to 128 cells/mm^3 in 3 months); 3) a short temporal gap (2months) between HAART initiation and the disease worsening; 4) an inflammatory reaction causing the worsening of previous lesions and the appearance of new lesions revealed in neurologic imaging studies; 5) the exclusion of other differential diagnosis through negative results on specific tests; and 6) clinical and radiologic improvement without particular empiric therapies. Yet, an incomplete anti-toxoplasma medication compliance could lead to a worsening of the first disease - still we consider that if it happened, a marked worsening of all the previous lesions would be present

instead of the appearance of new lesions while other were stabilized, and also the patient stated that he had correctly took his medication.

Regarding the pathogen causing the CNS-IRIS, although our perception is to consider toxoplasma the causative agent, we cannot exclude a CNS-IRIS related to HIV itself. The most common cause of myelopathy in AIDS patients is vacuolar myelopathy caused by HIV (even if asymptomatic) [30,31] and only few cases of myelitis by toxoplasma were described [19 and reference therein,25,26]. The appearance at MRI of a vacuolar myelopathy caused by HIV could, however, be quite different (although it is more frequent at thoracic medulla, also the portion of medulla more affected in this case); usually it is more diffuse, and with atrophy [31] characteristics not present herein. Although rare, a reduced number of reports described the occurrence or worsening of HIV-associated neurocognitive disorder after HAART initiation [7 and reference therein], but as far as we know, cases of HIV-associated myelo-CNS-IRIS have not been described. On the other hand, at least one case of toxoplasma-associated myelo-CNS-IRIS has already been described [19]. We consider that an under-diagnosed involvement of the spinal cord by *T. gondii* could be present in this patient when the symptoms only revealed brain involvement, and that the inflammation caused by IRIS rendered the spinal cord involvement clinically evident.

A critical question is if it could be a miscellaneous-IRIS: a toxoplasmic cerebral IRIS plus a HIV medullar IRIS. It seems to us a quite unlikely coincidence. A negative CSF HIV load could help to dismiss this diagnosis although a positive value would not however confirm the diagnosis since we know that HIV could be present in CSF and not causing cerebral or medullar lesions [32].

A excisional brain or medullar biopsy could probably dispel any doubt; the demonstration of an inflammatory perivascular infiltrate dominated by $CD8^+$ T cells and macrophages that is typical of IRIS triggered by not only HIV, but also by toxoplasma, would provide evidence about the existence of an IRIS [7]. The demonstration, on the spinal cord, of a granulomatous inflammation and of scarce toxoplasma pseudocysts (or bradyzoites) surrounded by inflammation, and an attenuated immunoperoxidase reaction meaning sequelae of effective antitoxoplasmosis therapy, would dissipate any doubt about the causative agent of myelo-IRIS. However, as this is most probably a case of paradoxical-IRIS, and given that HAART was initiated one month after the onset of specific anti-toxoplasma therapy, the chance to detect replicative forms of toxoplasma – tachyzoites would be very low, and we very probably would not see any toxoplasma pseudocysts nor immunoperoxidase reaction, that is what happened in the clinical

case reported by Cabral *et al* [22]. In the other four cases of paradoxical toxoplasmosis-IRIS described in the literature [19,23,24], however, tachyzoites have been found in brain or in spinal cord biopsies what could be explain by the simultaneous onset of anti-toxoplasma and antiretroviral therapies. The demonstration of vacuolation in the spinal white matter in association with a few lipid-laden macrophages within the vacuoles or the myelin sheath are neuropathological findings of HIV-related vacuolar myelopathy [30] but in a case of HIV-myelo-IRIS, those findings could also not be present as the specific treatment (HAART) has already been taken for three months.

The decision to postpone the biopsy, as soon as the patient showed signals of improvement, was based on inherent risks for the patient of the invasive procedure. The use of MRI imaging in association with the clinical and laboratory data, and a good clinical judgment prevented the performance of an unnecessary and perilous brain or medullar biopsy.

During the probable CNS-IRIS event, we also did not use corticosteroid therapy because we were frightened that an undiagnosed condition could worsen due to the impairment of immune function and also that it could facilitate the occurrence of other opportunistic infections. As our patient did not reveal severe manifestations and the efficacy of corticosteroids in IRIS is not consensual, we choose not to use them. Accordingly, in all cases of CNS-IRIS related to toxoplasmosis described, but one [19], the clinical outcome was favorable with no corticosteroid treatment. Concerning HIV vacuolar myelopathy and myelo-HIV-IRIS, as far as we know, data concerning the use of corticosteroids in those conditions do not exist. In HIV vacuolar myelopathy, the prognosis is often bad even continuing the specific therapy (HAART) what did not happen in our case, the patient's health state improved with the maintenance of HAART.

To our knowledge, this clinical case of paradoxical toxoplasmosis IRIS is the sixth reported [19,20,21,22,23,24], and only the second with spinal cord involvement [19].

An early diagnosis and therapy of HIV infection will avoid severe immunodeficiency and opportunistic infections and, consequently IRIS related conditions after HAART onset. A very close monitoring is recommendable in cases of CNS-IRIS, including when CNS toxoplasmosis is present. Although both the biopsy and steroids therapy might be of use and need to be considering for these patients, they were both not necessary in the present case.

Concerning the brief analysis of Treg and its subsets performed at several time-points of the clinical case and controls, the more pronounced changes, Treg and Treg subsets related, in

the reported case comparing to controls, indicate that probably changes in this $CD4^+$ T cells subset contributed for IRIS.

The peak of IRIS-related symptoms in the reported case, however coincides with the peak in Treg percentages. Unfortunately, the characterization of Treg in naïve and memory does not allow us to infer about the suppressive function of Treg. It will be of paramount importance to well characterize in terms of function Treg subsets since these Treg could be non-suppressive. It will be also of importance to determine the responsiveness of the new $CD4^+$ T cells to Treg since the Treg detected could be functional but trying to counter $CD4^+$ T cells that are in a state of refractoriness. Another hypothesis would be that the observed peak of Treg at third month was a feedback reactive increase in Treg trying to counteract the exaggerated inflammation. A high dynamic rate of conversion of Treg (naïve to memory, and on the memory subset from activated to non-supressive and probably consequently, death by apoptosis [33]) could probably explain both the changes in the Treg subsets and the inability of those cells to counterbalance the strong immune response against the microbial agent, in this case, *Toxoplasma gondii*.

2.4.5. Bibliography

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Chapter 3

Final Remarks, General Discussion and Conclusions

3.1. Final Remarks, General Discussion and Conclusions

The Acquired Immunodeficiency Syndrome (AIDS) is a disease of the human immune system caused by a virus – the human immunodeficiency virus (HIV). AIDS was first recognized in 1981 [1,2] and dramatically evolved to a global pandemic. In 2012, globally, an estimated 35.3 (32.2–38.8) million people were living with HIV, there were 2.3 (1.9–2.7) million new HIV infections, and the number of AIDS deaths was 1.6 (1.4–1.9) million [3].

In the beginning, in the 1980s and early 1990s, AIDS was a virtual death sentence; patients had to endure numerous hospitalizations to treat opportunistic infections before they eventually succumbed to the illness. Those terrible times still remain in the memory of patients' family members and health care professionals who, at the time, began to take care of patients suffering from this disease.

Fortunately, huge advances were made until now in this field, being the emergence of drugs with potent antiretroviral activity, and their use in combination (highly active antiretroviral therapy or HAART), started from 1996, the most important landmark on the evolution of the disease that had markedly reduced morbidity and mortality AIDS-related. HIV infection is now considered a chronic disease easy to stabilize, at least, when patients are early diagnosed, where therapy and laboratory support are available, and when patients adhere to treatment and health care. There is a striking contrast between how we felt as physicians in the 1980s and early 1990s and the confidence we experience today in the increase over time of the expectancy and quality of life of patients suffering from this disease, making it closer and closer to ones of those not infected by HIV.

Some issues related to this infection, even if patients are maintained many years under effective therapy, however, remain to be solved and are still a concern for patients and their physicians: the impossibility to achieve a complete eradication of the virus; the persistence of a residual immune activation and inflammation accounting for faster aging and for a number of non-AIDS-related co-morbidities [4,5]; and also the inability of some patients to reconstitute their immune system maintaining an increased risk of suffering from AIDS-related and non-AIDS-related complications and death [6-10].

A percentage of HIV-patients that initiate HAART (ranging between 15-40% depending on the study), although being virological suppressed during several years, do not reach a normal (700-1100 cells/mm^3), a near normal (> 500 cells/mm^3) or, at least, a satisfactory $CD4^+$ T cells count (a number higher than a threshold of 250-350 cells/mm^3, a level that would enable

patients to be protected from more severe opportunistic infections), and therefore are more prone to suffer from AIDS-related and non-AIDS-related complications and death [6-10]. These patients have been referred in many ways; as immunological non-responders or discordant, or inadequate or incomplete responders. There is no agreement on the definition of immunological response failure and several definitions have been reported including a diverse range of patients – some authors give more importance to the value of $CD4^+$ T cells reached, others to the increase in percentage or to the difference (in numbers) compared to baseline level. Several scientific explanations may support that incomplete recovery (reviewed in Subchapter 1.1) [6-16]. Immunological response failure is a subject which is hard to deal with at a clinical basis, patients do not understand why their $CD4^+$ T cells count stop to increase and do not reach a optimal level although they correctly adhere to medication. And also for physicians, it is difficult to explain to their patients the reasons for that to happen, to prove and demonstrate why it happens, and above all, to offer effective solutions to protect patients from the increased risk for AIDS-related and non-AIDS-related complications and death.

T regulatory T cells (Treg) (reviewed in Subchapter 1.2) are a subset of $CD4^+$ T cells that are able to suppress potentially excessive immune responses, therefore being essential for the maintenance of the immune system's equilibrium. The role of these cells has been for long discussed as potentially important not only in HIV infection progression but also in immune recovery under effective HAART. Moreover, these cells have also been proposed to be an important factor/intervener in the immune reconstitution inflammatory syndrome (IRIS). Despite a great number of reports concerning the role of Treg in the HIV infection, their precise role remains to be clarified. A huge amount of uncertainty and ambiguity subsist concerning the effective role of Treg in the HIV infection scenario. Two mainly opposite roles in this situation have been endorsed to Treg: a beneficial role by dampening the immune activation/inflammation [17-19]; and a detrimental role by fading HIV-specific $CD4^+$ and $CD8^+$ T cell responses [20-22]. Also, supporting a beneficial effect of this T cell subset, it has been suggested that Treg could suppress HIV-replication in conventional $CD4^+$ T cells [23]. On the other hand, it has also been advocated that Treg may inhibit lymphopenia-induced proliferation [24,25], can lead to a more intense gut microbial translocation accounting for the augmented immune activation [26,27], or may account for exacerbated fibrosis of lymphoid tissues (by increasing levels of TGF-α1 and so exacerbating collagen deposition) jeopardizing therefore the immune reconstitution process [28].

The uncertainty found in the numerous papers about the real role of these cells in HIV infection progression could be driven by several factors such as: 1) Treg may be beneficial or detrimental depending on the disease stage; 2) studies may have been characterizing and measuring Treg with distinct methods by using different markers/gating strategies; 3) it is not yet clarified if HIV itself or HIV infection scenario modifies the potency of suppressive activity of Treg; 4) also, it remains ill-defined what is the dynamic and redistribution of the different Treg subsets that have distinct suppressive activity and probably different roles in HIV infection [29-31]; 5) it is also not known how and when plasticity of Treg can occur leading to modifications or even loss of their suppressive function and even became pro-inflammatory cells; and 6) it is not known if the relationship between Treg and beneficial or detrimental effect on HIV infection disease depends more on Treg absolute number or Treg percentage; it could be that, for example concerning the beneficial effect of Treg decreasing immune activation, the most important would be absolute numbers instead of Treg percentages [19,32,33].

It is of paramount importance to know exactly what the Treg role in HIV infection is, so that with the possibility to manipulate them, an improvement in HIV infection disease management could be achieved.

Having all this in consideration, and the fact that the majority of the studies addressing Treg in HIV infection are cross-sectional or small longitudinal studies, we endeavor a work involving HIV-infected patients selected from the hospital where they underwent medical care. Thereby, the main goals of this thesis were to know: i) if and how, in untreated HIV-infected patients, Treg and their subsets are disturbed and how this is related to disease severity; ii) how, in HIV-infected patients under HAART, Treg and their subsets behave over time; iii) if Treg influence the immune recovery in patients under HAART; and also iv) if the deregulation of Treg or their subsets were implicated in IRIS.

Thus, we constituted 2 cohorts; a cohort for a single-moment evaluation and a cohort for a prospective longitudinal evaluation of Treg as well as several other parameters. A control group composed by healthy individuals not HIV-infected was also constituted (these cohorts were described at Subchapter 2.1). The constitution of these cohorts initiated a more profound and serious collaboration between the hospital (HJUU)/the clinicians/the patients and the research laboratory (ICVS). For the moment these studies are completed or close to be completed but the material and information generated, as well as the dynamic between the hospital and the ICVS will allow several other studies to be performed. These studies present in this thesis were

performed with the results of the evaluation of 53 patients from the cross-sectional cohort (all of them aviremic under effective HAART for more than a year) (described at Subchapter 2.2), and the longitudinal study (described at Subchapter 2.3) that presented the results of the evaluation of the first two years under treatment of 81 patients (patients adherent to therapy and aviremic at the 24 month time-point). Moreover, one of the patients of the longitudinal cohort presented IRIS toxoplasmosis-related (presented in the subchapter 2.4), allowing us to analyze Treg and Treg subsets dynamics of this patient before, and throughout this pathologic process.

It is worth noting that as far as we know, and concerning Treg cells in the HIV infection context, our cohort is the longitudinal cohort with the highest number of patients for such a period (from the 100 patients recruited, 77 are still in the study, 83 completed two years, 59 completed three years, and 11 completed four years of follow-up).

Among chronically HIV-infected untreated patients, the ones in advance stage of the disease present higher Treg percentage and higher diversity of these values

Some disagreement exists on the reported evaluation of Treg percentages from HIV-infected patients. However, the majority of the studies show that the absolute number of Treg is decreased in HIV-infected individuals, but in a lower proportion than the whole $CD4^+$ T cell population, resulting in a increased blood Treg percentage on $CD4^+$ T cells comparing to healthy controls [20,32,34,35, reviewed in 36,37].

Taking into account our results we consider that part of the contradictory data published concerning Treg percentages (Treg percentages similar or lower than healthy individuals) [21,29,30,38,39] may be explained by the use of different Treg markers and/or distinct gating strategies [21,39], from the use of FOXP3 mRNA measured by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) [21] in T cells to define Treg instead of flow cytometry (a distinct FOXP3 mRNA expression per cell may occur thus do not enabling the correct evaluation about the number of Treg) [21], from different biological samples analyzed (whole blood, fresh peripheral blood cells, or cryopreservated blood [29,39]), or also from heterogeneity in the HIV population studied between studies (acute infected, untreated long-term viremic, or aviremic under treatment patients, and elite controllers - EC - or long-term non-progressors patients - LTNP) [30,38].

In our study, we employed the currently used and apparently the most reliable multicolor antibodies cocktail for Treg markers - $CD3^+CD4^+CD127^{low}CD25^{high}FOXP3^+$ [40-43] (although the

purity of Treg reached using such strategy remains under debate [44]). Also, all the samples were processed on the day of the collection so all the analysis was performed on fresh blood and in similar conditions for all individuals at all time-points. And, probably, more importantly, the study included a great number of individuals (n=81) that were all chronically HIV-infected without HAART (baseline) and presenting a wide range of $CD4^+$ T cells (however, all of them presented progressive disease and criteria to initiate HAART considering the National and International Guidelines [45-48]).

We found that these 81 patients displayed a significant higher Treg percentage median comparing to control individuals. Interestingly and looking to the data more behind the median level, we noticed that Treg percentage range was much wider in HIV patients than healthy controls. So, even being chronically infected and naïve for treatment, the values for Treg percentage were extremely diverse. When patients were stratified according to their nadir counts of $CD4^+$ T cell count, we noticed that in the strata of those with lowest nadir (<200 cells/mm^3), those findings (higher Treg median and wider range of Treg percentages) were much more evident than when the data from all individuals were analyzed as a whole. In fact, only in this strata the Treg baseline median was significantly higher than that of control group. Thus, a link between high Treg percentage (and also high range of values) and more advanced disease seems to exist.

Previous studies had already showed an inverse relationship between Treg percentage and $CD4^+$ T cells counts [14,24,34,49-51], and even some authors [19,52] had already noted a high variability inter-individual in Treg percentages. But, in our study when we stratified the sample by $CD4^+$ T cells, we showed clearly negative correlation - as lower was the $CD4^+$ T cells counts at baseline, the higher the Treg percentage and the variability between individuals. The reason why HIV-infected patients in advanced stages of the disease, have so distinct Treg percentages remains to be elucidated. We are hopeful that through the analysis of all the patients and their clinical and laboratorial data collected from this cohort, we can be able, in the future to clarify, or at least provide more information that will contribute to clarify, the mechanisms behind this phenomenon.

Concerning to the link between high Treg proportions among $CD4^+$ T cells and low $CD4^+$ T cells; although it might indicate that Treg play a detrimental role, or that high levels of Treg are, at least, not an advantage for a good evolution of the disease, the nature of our study does not allow us to conclude that.

The questions about the real role of Treg in the progression of HIV infection cannot be answered by a static view of the Treg in a given moment of the disease evolution as we did when we analyzed patients on the day they initiated HAART. With such analysis it is not possible to determine whether the alterations detected existed prior to the progression of the disease, or emerged after the progression of the disease by a negative feedback loop trying to control the disturbances generated by the disease. To answer this question it will be necessary to perform in the future a longitudinal study including patients since the moment of infection and during the progressive disease without treatment to clarify the precise role of Treg in the progression of the disease. Also, studies aiming the interrelationship between Treg and specific anti-HIV immune response and between Treg and immune activation over time will be necessary for that clarification. Only in that way we will be able to understand the temporal order of events and characterize the direction and the magnitude of potential cause-and-effect relationships between Treg and HIV disease progression. We must not forget, however, that such a study could face ethical problems and couldn't certainly be done up to an advanced stage of the disease. Currently, it is advocated that HAART begins earlier and earlier in the disease course once that was shown to be beneficial to patients [45].

In addition to causing alteration on the proportion of total Treg among the $CD4^+$ T cells, HIV infection disturbs the subsets distribution within Treg

Concerning the analysis of Treg subsets in untreated HIV-infected patients, a disturbance in Treg subsets homeostasis was consistently found by other studies [29-31]. However, the heterogeneity in the HIV population studied, the heterogeneity of the characterization methods to identify Treg subsets [53-55] and/or different proportions utilized (Treg subsets on Treg versus Treg subsets on $CD4^+$ T cells) by the authors of the diverse studies turn the comparison not straightforward. Simonetta *et al* [29], and Serana *et al* [30] found that the effector ($CD45RA^-$ $FOXP3^{high}$) or central memory ($CD45RA^-CCR7^+$) Treg cells, respectively, were the ones more affected in untreated HIV-infected patients. Contrary, Zhou *et al* [31] found to be the naïve Treg those more affected in the patients (although they used a different approach to identify Treg subsets). We also used a different Treg subset identification strategy based on CD45RA expression that allowed us to identify naïve ($CD45RA^+$) Treg, and memory ($CD45RA^-$) Treg [55] and contrary to other authors we used Treg proportions on total Treg instead of Treg subsets on $CD4^+$ T cells. We found that in chronically untreated HIV-infected patients, in those most

immunosuppressed (<200 $CD4^+$ T cell/mm^3), the percentage of naïve Treg on total Treg are lower comparing to healthy controls. As already mentioned above, although an accurate comparison between studies is not possible, one explanation to the differences found could be the more advance stage of the disease in our patients and in the ones evaluated by Zhou [31] than those analyzed in the studies of Simonetta [29] and Serana [30]. The percentage of cycling Treg ($Ki67^+$ Treg) on Treg in the most immunosuppressed untreated HIV-infected patients (<200 $CD4^+$ T cell/mm^3) was also different from the one in healthy individuals, being higher in patients. Other studies have also found this disturbance [31,56].

It has been advanced that phenotypically and functionally distinct Treg subsets may exert different suppressive effects, or even being ones beneficial and others deleterious in the HIV infection context [29,57]. The accurate characterization of Treg subsets, rather than an evaluation of the total Treg population, may lead to a deeper understanding of the Treg role in HIV infection.

HAART tend to normalize total Treg proportion on $CD4^+$ T cells but not the high variability inter-individual and the proportions of the Treg subsets

According to most studies, providing that reliable Treg markers/gating strategy were used, Treg percentage value tends to normalize reaching, after a certain time of treatment, values similar to the ones of controls [32,34,50,58]. Some of those studies showed a transient early increase of Treg percentage just after the therapy onset (probably explained by the liberation of Treg from lymphatic tissues to periphery due to the down-regulation of adhesion molecules associated in turn with a decline in viral replication) and before the consistent decrease [34,50]. Some studies, however, postulated that Treg expansion persists despite viral control under HAART [20,35,49,59,60]. Explanations for that discrepancy have already been proposed in subchapter 2.3: heterogeneity of patients included (treated but not aviremic, short time vs long-term under treatment, low nadirs vs high nadirs at baseline), or suboptimal combination of Treg cell markers.

In our longitudinal study we found that upon therapy initiation, in those patients more imunosupressed (<200 $CD4^+$ T cells/mm^3), who presented at baseline a Treg median percentage higher than the one of healthy individuals; Treg percentage progressively decreased reaching for the majority of the patients values within the normal range. We did not find the initial transitory

increase after HAART initiation suggested by others [34,50]. Also, the Treg percentage range that was wider in HIV patients tended to become progressively more restricted along the treatment.

Both in our cross-sectional study (involving patients under treatment for several years and at least 1 year) and in 24M time-point of the longitudinal study, we found that, comparing to a control group of healthy individuals, although Treg median percentage were not different from the one of healthy controls, a heterogeneous distribution of Treg percentages among HIV-infected individuals under HAART persists upon several years of therapy (in the cross-sectional study, the media time under HAART was of 6,5 years, ranging from one to 14 years) and with 24M of therapy (longitudinal study). And, although the median Treg percentage was not significantly different, the number of patients with a Treg percentage≥10% was sign ificantly superior to those within the healthy group. In our cross-sectional study, we tried to see if this highly diversity in Treg percentages values was explained by some factor such age, years under HAART, infection by hepatitis C virus, or overall immune activation, but no impact of these variables in Treg percentage was found. Also, all the patients were virological suppressed not suffering from opportunistic infections or other AIDS-related conditions that could explain so different values.

Also the alterations seen in Treg subsets in patients at baseline of the longitudinal study (lower naïve Treg and high proliferation of Treg than ones of healthy controls) persisted despite 24M under HAART in those patients more imunossupressed (<200 $CD4^+$ T cells/mm^3). Stratifying by nadir values and following-up patients for longer periods will be necessary to determine if more time under HAART would completely restore Treg homeostasis in all patients, only in those less imunosupressed or in none of them (being those alterations irreversible despite many years under HAART). This information could help to define cut-offs for HAART initiation.

The studies already referenced [29-31] found a tendency for HAART to promote the normalization of Treg subsets, although a persistent lower value of activated/effector Treg cells count was found in the study of Simonetta *et al* [29], and a persistent high Treg proliferation was found by Zhou *et al* [31].

Treg percentages at treatment initiation do not seem to represent an additional variable affecting T cell reconstitution

The role of Treg in the immune recovery process in HIV-infected patients upon effective HAART remains to be elucidated. Some cross-sectional studies [14,24,56,61] including our

cross-sectional study suggested a negative role of Treg percentage in the immune reconstitution after HAART, but also the contrary was shown [16].

A cross-sectional study provides only a static view in a given moment of the immune recovery. A longitudinal cohort study involving a population of patients, from the moment they initiate HAART, followed-up at the same several time-points of their evolution, and evaluated using a gating strategy for a reliable set of Treg and functionally distinct Treg subsets markers, was needed to better understand the temporal order of events. Only with such study, it was possible to determine the definition of the direction and the magnitude of potential cause-and-effect relationships between factors, namely Treg and its functional subsets, taking place in the immune reconstitution process just before HAART onset and during the treatment. The majority of the studies have been performed comparing groups of patients with control groups but they are not longitudinal studies. Longitudinal studies concerning this matter are few and include a small number of patients [31,32,34,49,50,51,59,60,62].

Concerning to the probably link found in our cross-sectional study between higher Treg percentages and a poorer immune reconstitution in a given time-point, we hypothesized that a high percentage of Treg at the onset of HAART could be an additional predictor of worse immune reconstitution. This hypothesis was not confirmed, at least after 24 months of treatment in our longitudinal study. However, a correlation analysis showed that high Treg percentages at baseline correlated with lower $CD4^+$ T cells count at the 24 months time-point. Nevertheless, we found that individuals with higher Treg frequency at baseline have also lower $CD4^+$ T cell counts at baseline. And those patients consistently showed a lower $CD4^+$ T cell counts throughout the follow-up period (as it would be expected according to $CD4^+$ T cell counts at baseline). Performing a multivariate analysis, in fact, we found that although Treg percentage at baseline seemed to be a good negative predictor of immune recovery, it lost its power when we introduced the nadir of $CD4^+$ T cell in the model, showing that the high Treg percentage is linked to a low $CD4^+$ T cell count, and what predicts the number of $CD4^+$ T cells after a given period of time is the $CD4^+$ T cells count at baseline and not the Treg percentage at baseline. Thus, Treg percentage seems to be no more than an indirect negative predictor of $CD4^+$ T cells reconstitution as its value is linked to of $CD4^+$ T cells count that itself is the direct predictor of the $CD4^+$ T cells count after a given period under effective HAART.

Apparently, the results found in our longitudinal study failed in demonstrating a link between high Treg and poor immune reconstitution (under HAART) suggested by our previous

cross-sectional study. However, while in the cross-sectional study, we analyzed patients with $CD4^+$ T cell counts under 500 cells/mm^3 at baseline, and patients who were for a long time under HAART (media of time under HAART=6,5 years; range: 1–14 years), in the longitudinal study, we analyzed patients showing a diverse range of $CD4^+$ T cell counts, at baseline (ranging from 8 to 1033 cells/mm^3), and after only 2 years under HAART. We consider of relevance to keep the follow-up of these patients at least up to the 5 years under HAART to clearly understand if the disturbed percentage of Treg among the $CD4^+$ T cells is an additional factor contributing for incomplete immune reconstitution.

Treg and Treg deregulation seem to be involved in IRIS

IRIS's immune-pathogenesis is poorly understood; it is characterized by an exaggerated immune-response against a specific pathogen (already present when HAART was initiated) that occurs after the restoration of the host defenses [63]. Treg are cells that are able to suppress/reduce exacerbated immune responses being essential for the maintenance of the immune system's equilibrium. Thus, It has been suggested that Treg may be involved in IRIS pathogenesis: i) due to a rapid rebound of conventional T cells after HAART initiation that is not followed by a parallel raise of Treg, which could lead to a decreased on Treg percentages and thus to an unbalanced proportion of $CD4^+$ T conventional/Treg [51]; ii) although present, Treg might be dysfunctional [58,64,65]; or iii) the new conventional $CD4^+$ T cells might be refractory to Treg function [51].

Taking advantage of the fact that one of the patients of our longitudinal cohort developed toxoplasmosis-paradoxal IRIS, we analyzed Treg and Treg subsets dynamics before, throughout and after that pathologic process (described at subchapter 2.4). A small control group was also composed to allow us to search for differences and consistent pattern in our case that would help to understand the pathologic condition. We noticed a high variability inter-individual between all the patients (the case and other patients). However a more pronounced variation of Treg/Treg subsets during IRIS was noticed in our case which seems to indicate that a deregulation in Treg/Treg subsets could be involved in IRIS. Unexpectedly, the peak of IRIS-related symptoms and the peak of Treg percentage among $CD4^+$ T cells occurred simultaneously. We also noted that at that peak, almost all of Treg were memory Treg. It remains to know about the suppressor function of those cells. To better clarify this event, it would be of central importance to identify and quantify the phenotypically and functionally distinct Treg subsets. Unfortunately we

characterized the different Treg subsets based only on CD45RA expression that allowed us to divide Treg between naives and memory but do not allowed us to know about the functional potency of the Treg subsets involved. The use of the expression of CD45RA or CD45RO combined with FOXP3 (low vs high) would allow us to know better about the functional power of the subsets present in our case. Memory Treg comprises both activated Treg (those supposedly more suppressive – $CD45^{-}FOXP3^{high}$) and nonsupresive Treg (those supposedly without suppressive function – $CD45RA^{-}FOXP3^{low}$) [53]. It will be also important to test the responsiveness of T cells to these Treg to understand all the factors involved in this process. Another hypothesis would be that the observed peak of Treg at the third month was a feedback reactive increase in Treg trying to counteract the exaggerated inflammation.

Final remarks

Despite the occurrence of major breakthroughs in recent years to the understanding of the role of Treg in HIV disease, much remains to be investigated before it will be possible to manipulate these cells in the right direction to use in clinical practice in benefit of our patients suffering from this disease.

We have given, with this work a small contribution to the knowledge of the relationship Treg/HIV infection. In addition we think that we can still do a lot with the cohorts that we gathered. We think that to characterize the subsets of Treg, know them in terms of function, distribution and dynamics in the context of this disease will be of extreme importance. Try to understand why the higher but also more varied percentages presented by patients in more advanced stages of the disease occur will be also one of our next goals. We may further deeply study the relationship between Treg (and its subsets) and the appearance of IRIS in patients initiating HAART in advanced stages of the disease. Perceive as a lack of standardization (or his delay) after HAART, either in variability of the percentages of Treg, either in the proportions of different subsets may be important in immune reconstitution process under HAART will certainly help us to improve medical care to our patients. HIV causes a steady imbalance mainly at the level of the cell-mediated immunity being Treg and its subsets also affected. These alterations and disturbances may be irreversible even under HAART mainly if the therapy onset is delayed. These findings further reinforce the need to initiate HAART early in the course of the disease.

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Annexes

Annexes

Annex 1

Universidade do Minho

TERMO DE CONSENTIMENTO INFORMADO

Declaro que o sangue que me foi colhido, poderá ser utilizado para investigação no estudo "Reconstituição e Homeostasia do Sistema Imunitário e na Infecção por HIV", que me foi explicado pelo técnico abaixo assinado, poderá ser utilizado neste e apenas neste estudo ou outro da mesma instituição exclusivamente para efeitos de investigação cientifica.

Fui informada/o de que a amostra de sangue não será identificada, tendo apenas como informação o género e a idade, pelo que os resultado não me serão divulgados.

Fui também informada/o de que, mesmo concordando agora, poderei no futuro retirar esta autorização, e também de que, qualquer que seja a minha decisão, agora ou no futuro, não serei prejudicado no meu direito à assistência na doença.

Nome completo do dador: _____

Local: Instituto de Investigação em Ciências da Vida e Saúde, Escola de Ciências da Saúde, Universidade do Minho, Campus Gualtar

Data: _____/ _____/ _____

Assinatura do dador: _____

Assinatura do Técnico: _____

Annex 2

centro hospitalar
do Porto

Hospital Santo António | Hospital Maria Pia | Maternidade Júlio Dinis | Hospital Joaquim Urbano

Largo Prof. Abel Salazar
4099-001 PORTO
www.hgsa.pt

Exm.ª Sr.ª
Dr.ª Ana Maria Lacerda Morgado Aboim Horta
Serviço de Doenças Infecciosas
Unidade Hospital Joaquim Urbano

ASSUNTO: Trabalho Académico – Doutoramento - "Reconstituição e homeostasia do sistema imune na infecção por VIH"- N/ REF.ª 127/12(NA-DEFI/089-CES)
- Adenda

O Conselho de Administração do CHP **autoriza** a adenda ao estudo de investigação acima mencionado nesta Instituição, no Serviço de Doenças Infecciosas da Unidade Hospital Joaquim Urbano, sendo Investigador Principal, a Dr.ª Ana Maria Lacerda Morgado Fernandes de Carvalho de Aboim Horta.
A adenda ao estudo de investigação foi previamente analisada pela Comissão de Ética para a Saúde, bem como pela Direcção Clínica, tendo obtido Parecer Favorável.

Cumprimentos,

CONSELHO DE ADMINISTRAÇÃO
Dr. SOUSA ALEGRO — Presidente
Dr.ª ÉLIA GOMES — Vogal Executiva
Dr. PAULO BARBOSA — Director Clínico
Dr. PORTO GOMES — Vogal Executivo
Enf.º EDUARDO ALVES — Enfermeiro Director

* Em todas as eventuais comunicações posteriores sobre este estudo é indispensável indicar a nossa ref.ª.

Annex 3

centro hospitalar do Porto

Hospital Santo António · Hospital Maria Pia · Maternidade Júlio Dinis · Hospital Joaquim Urbano

Largo Prof. Abel Salazar
4099-001 PORTO
www.chporto.pt

APRECIAÇÃO E PARECER PARA A **ADENDA** AO TRABALHO ACADÉMICO - DOUTORAMENTO

Título: "Reconstituição e homeostasia do sistema imune na infecção por VIH"		Ref.ª: 127/12(NA-DEFI/089-CES)
Protocolo/Versão: **- Adenda**		Investigador: **Dr.ª Ana Maria Lacerda Morgado FC Aboim Horta** **Hospital Joaquim Urbano**

DIRECÇÃO DE ENFERMAGEM:	DIRECÇÃO CLÍNICA:
☒ NÃO SE APLICA	☒ PARECER FAVORÁVEL
☐ PARECER FAVORÁVEL	☐ PARECER NÃO FAVORÁVEL
☐ PARECER NÃO FAVORÁVEL	
Data:	Data: 27/3/12
	Dr. Paulo Barbosa (Director Clínico)

Em conformidade. Pode ser autorizado

19 MAR. 2012

Prof. Doutora Margarida Lito
Directora do DEFI

Annex 4

Protocolo – Doutoramento

Dinâmica da reconstituição e homeostase do sistema imune em indivíduos com infecção VIH/SIDA - tratados

Autocolante do doente

Data da colheita:_____

Identificação do doente:_____

História anterior

Modo de transmissão: _____

Data provável de Infecção: _____

Data do diagnóstico: _____

Infecções/patologias oportunistas:

Data dos diagnósticos/ quais:

_____

_____

_____

_____

_____

_____

_____

Outros Antecedentes: _____

_____

_____

Co-infecção VHC/VHB: _____

TARV actual (sim/não/qual): _____

Regular? _____

Data de início do esquema actual: _____

Datas e esquemas anteriores, motivo da mudança:

_____

_____

_____

_____

_____

_____

_____

Últimos CD4 e CV VIH (data e valores):

_____

Outras notas:

Annex 5

Formulário de Consentimento Informado

Nome do Estudo: Reconstituição e Homeostasia do Sistema Imune na Infecção por VIH

Investigador Principal: Ana Aboim Horta

Convidamo-lo a participar num estudo de investigação com a duração de cerca de três anos.

Introdução

O VIH infecta e destrói essencialmente um determinado tipo de células de defesa do nosso organismo, as células T $CD4^+$ ou como são mais conhecidos, os CD4, impedindo assim o desenvolvimento de respostas de defesa apropriadas contra diversos agentes infecciosos. Desta forma, indivíduos infectados com o VIH, principalmente aqueles em que o número de CD4 se encontra abaixo de 200 células/mm^3 (o valor normal é de 700 a 1000/mm^3), são muito susceptíveis a diversas doenças infecciosas podendo adoecer e mesmo morrer devido a essas infecções. O aparecimento de medicamentos com actividade antirretrovírica potente (actividade contra o VIH), e o seu uso em combinação no tratamento da infecção pelo VIH, veio, a partir de 1996, reduzir de forma marcada a morbilidade e a mortalidade associadas a esta doença e aumentar a esperança de vida destes pacientes. Estes medicamentos impedem o vírus de se multiplicar e, assim, de destruir as células de defesa, ficando o doente capaz de evitar as infecções.

Quando temos uma infecção, o nosso sistema imune (as nossas defesas) entra em acção na tentativa de eliminar o agente infeccioso e, quando este objectivo é conseguido, tem que existir uma outra fracção do nosso sistema imune que faça parar essa defesa pois ela, em excesso, pode causar dano ao nosso organismo. Tem que haver sempre um equilíbrio entre as diversas fracções do nosso sistema imune e nestes doentes infectados por VIH, questões fundamentais vão surgindo: i) como é que o sistema imune destes pacientes recupera com a terapia antirretroviral após um tão grande desequilíbrio; ii) que tipo de homeostase ou equilíbrio é atingido; iii) como é que factores como o próprio indivíduo, a sua idade e o seu sexo ou o início da doença clínica afectam o novo equilíbrio do sistema imune.

Estudos recentes sugerem que durante as fases iniciais da terapia antirretroviral os pacientes, juntamente com um aumento dos CD4, mostram também uma maior predisposição para o desenvolvimento de respostas inflamatórias exuberantes contra infecções (respostas de defesa exageradas). Essas infecções podiam já estar identificadas e a ser tratadas com eficácia previamente ao início da terapêutica antirretrovírica mas, após o início desta, voltam a piorar (por

exemplo uma Tuberculose que já estava a ser tratada e em que o indivíduo já tinha melhorado e não apresentava sintomas, volta a piorar após o início da terapêutica antirretrovírica) ou podem ser provocadas por agentes ainda não identificados mas presentes antes do início da terapêutica antirretrovírica. Neste último caso, o baixo número de células de defesa, a apatia do sistema imune perante esses agentes infecciosos eram responsáveis pela ausência de sintomas e o doente apesar de já ter a infecção não a sentia. Ao iniciar a terapêutica, a recuperação das nossas defesas vai ser responsável pelo reconhecimento desses agentes, e por uma forte resposta à sua presença, o que provoca o aparecimento de sintomas de forma muito acentuada. Estas respostas exuberantes, apesar de permitirem o reconhecimento dessa infecção poderão ser perigosas.

A compreensão dos mecanismos base desta resposta imune exacerbada que acompanha a recuperação dos CD4 é pois essencial e, até à data, inexistente. Estes conhecimentos poderão ser utilizados para reconhecer os doentes em que isso poderá mais provavelmente ocorrer para, assim, podermos estar mais alertas e mesmo, talvez, evitá-lo.

Objectivo do estudo

Este estudo tem como objectivo estudar e recuperação imune através da caracterização de células e tentar perceber porque é que o nosso sistema imune, por vezes, não recupera de forma equilibrada. Pretende também identificar os agentes infecciosos contra os quais o nosso sistema imune, em recuperação, responde de forma mais exagerada.

Que doentes podem entrar no estudo?

Todos os doentes, infectados por VIH que necessitem de terapêutica antirretrovírica conforme as orientações internacionais e nacionais. Só poderão entrar no estudo os doentes que nunca fizeram terapêutica no passado ou o fizeram apenas durante um período inferior a três meses e há mais de um ano.

O que lhe irá ser pedido?

Se participar neste estudo, necessitará de fazer o seguinte:

- Visitar o seu médico no dia em que iniciar a medicação, uma vez por mês nos primeiros três meses, de três em três meses até ao fim do primeiro ano e depois, de quatro em quatro meses, até ao fim do estudo (o plano das visitas embora mais pesado, não difere muito do dos doentes que não entrarem no estudo, estes são vistos normalmente de 3/3 ou 4/4 meses podendo no início ter mais uma consulta intermédia).

- Tomar a medicação conforme a orientação do seu médico. A medicação que irá tomar será a mesma que tomaria caso não entrasse no estudo e segue as orientações internacionais.
- Se for mulher em idade fértil deverá concordar em utilizar métodos de controlo da natalidade aceitáveis durante o estudo. São considerados métodos contraceptivos aceitáveis os dispositivos intra-uterinos (DIU), o diafragma com espermicida, os preservativos e a abstinência sexual. Os contraceptivos orais isoladamente não são considerados aceitáveis de controlo da natalidade. Se engravidar durante o estudo deverá comunicar imediatamente ao seu médico pois a medicação poderá ter que ser ajustada.
- Nalgumas visitas poderá ter que vir em jejum de oito horas.

O que sucederá durante as visitas do estudo?

Quando comparecer no centro para as visitas, o investigador (o médico) ou outro pessoal do estudo envolvido (enfermeiro do estudo) poderão realizar um ou mais dos seguintes procedimentos:
- Questioná-lo sobre sintomas que tenha sentido.
- Efectuar um exame físico completo.
- Colher as amostras de sangue ou de urina requeridas para o estudo. Além das análises que faria mesmo que não entrasse no estudo, realizará colheita de sangue para caracterização das células de defesa e caracterização genotípica para identificação de factores genéticos de susceptibilidade genética a infecções (pode ter herdado uma maior sensibilidade às infecções).
- Nalgumas visitas poder-lhe-á ser pedida a realização de alguns exames imagiológicos ou outros no sentido de procurar outras infecções coexistentes.

Que benefícios poderei obter da minha participação no estudo?

Uma vez que visitarei o médico do estudo mais vezes do que o que seria realizado caso não entrasse no estudo, que serei observado e me serão realizados estudos analíticos/imagiológicos de forma mais regular do que o normal, poderão ser detectados infecções/problemas mais precocemente/atempadamente.

As informações colhidas durante o estudo poderão ajudar-me a mim e a outras pessoas no futuro.

Ao assinar este documento, declaro que:

Li este consentimento informado.
- Tive a oportunidade de colocar perguntas e obtive as respectivas respostas.
- Compreendo que a minha participação no estudo é voluntária e consinto-a.

- Posso optar por não participar no estudo ou abandonar o estudo em qualquer momento, desde que comunique o facto ao médico do estudo. Não serei penalizado nem perderei o direito a quaisquer benefícios que, noutras circunstâncias, me seriam devidos.
- Autorizo que os meus dados pessoais e de saúde sejam recolhidos e tratados, desde que a minha privacidade seja resguardada.

Irei receber uma cópia assinada deste consentimento informado

Nome completo do participante (maiúsculas):

_____

Local:_____Data_____/ _____/ _____

Assinatura do participante:

Nome do investigador (maiúsculas):

_____

Local:_____Data_____/ _____/ _____

Assinatura do investigador:

_____

Annex 6

HOSPITAL DE JOAQUIM URBANO
Ministério da Saúde Administração Regional de Saúde do Norte

Exmª Senhora
Drª Ana Aboim Horta
agaboim@hotmail.com

Hospital de Joaquim Urbano

Sua referência	Data	Nossa referência	Data	Processo
		168/CES	2.10.09	

ASSUNTO: **Pedido de autorização de realização do projecto de investigação "Reconstituição e Homeostasia do Sistema Imune na Infecção por VIH", da Drª Ana Maria Lacerda Morgado Fernandes de Carvalho Aboim**

Na sequência da reunião da Comissão de Ética do Hospital de Joaquim Urbano de 23 de Setembro de 2009, relativamente ao pedido de parecer sobre o projecto de investigação "Reconstituição e Homeostasia do Sistema Imune na Infecção por VIH", da Drª Ana Maria Lacerda Morgado Fernandes de Carvalho, depois de analisadas todas as alterações solicitadas foi dado parecer favorável à sua realização.

Com os melhores cumprimentos,

Porto e Hospital Joaquim Urbano, 2 de Outubro de 2009

A Comissão de Ética

Dr.ª Elizabete Maria das Neves Borges

Mod. 104-A

Rua Câmara Pestana, 348 – 4369-004 PORTO – Tel. 225 899 550 – Fax 225 106 160 – hju@hjurbano.min-saude.pt

Annex 7

Protocolo – Doutoramento

Dinâmica da reconstituição e homeostase do sistema imune em indivíduos com infecção VIH/SIDA

Autocolante do doente

Modo de transmissão: _____

Data provável de Infecção: _____

Data da última análise negativa: _____

Data da 1ª análise positiva: _____

Sintomatologia de primoinfecção? _____ Data: _____

Infecções/patologias oportunistas:

Quais, data de diagnóstico, data do início do tratamento:

_____

_____

_____

_____

Outros Antecedentes: _____

_____

Co-infecção VHC/VHB: _____

TARV e data (baseline):

_____

Protocolo de seguimento – Clínica/Exame Objectivo

	-30 d	Baseline	1°Mês	2°Mês	3°Mês	6°Mês	9°Mês	12°Mês
DATA								
Adesão	X	X	X	X	X	X	X	X
Astenia	X	X	X	X	X	X	X	X
Anorexia	X	X	X	X	X	X	X	X
Hipersud.	X	X	X	X	X	X	X	X
Febre	X	X	X	X	X	X	X	X
Alt.comport.	X	X	X	X	X	X	X	X
Cefaleias	X	X	X	X	X	X	X	X
Alt.visão	X	X	X	X	X	X	X	X
Disfagia	X	X	X	X	X	X	X	X
Dor torácica	X	X	X	X	X	X	X	X
Tosse	X	X	X	X	X	X	X	X
Expectoração	X	X	X	X	X	X	X	X
Hemoptises	X	X	X	X	X	X	X	X
Dispneia	X	X	X	X	X	X	X	X
Náuseas	X	X	X	X	X	X	X	X
Vómitos	X	X	X	X	X	X	X	X
Disfagia	X	X	X	X	X	X	X	X
Diarreia	X	X	X	X	X	X	X	X
Obstipação	X	X	X	X	X	X	X	X
Dor abdom.	X	X	X	X	X	X	X	X
Parestesias	X	X	X	X	X	X	X	X
Peso	X	X	X	X	X	X	X	X
Altura	X	X	X	X	X	X	X	X
TA	X	X	X	X	X	X	X	X
Pulso	X	X	X	X	X	X	X	X
Temp. ax.	X	X	X	X	X	X	X	X
Déficites focais	X	X	X	X	X	X	X	X
Sinais mening.	X	X	X	X	X	X	X	X
Candid. orof.	X	X	X	X	X	X	X	X
Aftas	X	X	X	X	X	X	X	X
Outra (cav.buc.)	X	X	X	X	X	X	X	X
Gânglios	X	X	X	X	X	X	X	X
Ex. ocular	X	X	X	X	X	X	X	X
Freq. Resp.	X	X	X	X	X	X	X	X
Alt. Pele	X	X	X	X	X	X	X	X
Qual	X	X	X	X	X	X	X	X
Ausc. Pulm	X	X	X	X	X	X	X	X
Ausc. Card	X	X	X	X	X	X	X	X
Palp. Abdom.	X	X	X	X	X	X	X	X
Alt. MS	X	X	X	X	X	X	X	X
Alt. MI	X	X	X	X	X	X	X	X

	16° Mês	20° Mês	24° Mês	28°Mês	32°Mês	36°Mês
DATA						
Adesão	X	X	X	X	X	X
Astenia	X	X	X	X	X	X
Anorexia	X	X	X	X	X	X
Hipersud.	X	X	X	X	X	X
Febre	X	X	X	X	X	X
Alt.comport.	X	X	X	X	X	X
Cefaleias	X	X	X	X	X	X
Alt.visão	X	X	X	X	X	X
Disfagia	X	X	X	X	X	X
Dor torácica	X	X	X	X	X	X
Tosse	X	X	X	X	X	X
Expectoração	X	X	X	X	X	X
Hemoptises	X	X	X	X	X	X
Dispneia	X	X	X	X	X	X
Náuseas	X	X	X	X	X	X
Vómitos	X	X	X	X	X	X
Disfagia	X	X	X	X	X	X
Diarreia	X	X	X	X	X	X
Obstipação	X	X	X	X	X	X
Dor abdom.	X	X	X	X	X	X
Parestesias	X	X	X	X	X	X
Peso	X	X	X	X	X	X
Altura	X	X	X	X	X	X
TA	X	X	X	X	X	X
Pulso	X	X	X	X	X	X
Temp. ax.	X	X	X	X	X	X
Déficites focais	X	X	X	X	X	X
Sinais mening.	X	X	X	X	X	X
Candid. orof.	X	X	X	X	X	X
Aftas	X	X	X	X	X	X
Outra (cav.buc.)	X	X	X	X	X	X
Gânglios	X	X	X	X	X	X
Ex. ocular	X	X	X	X	X	X
Freq. Resp.	X	X	X	X	X	X
Alt. Pele	X	X	X	X	X	X
Qual	X	X	X	X	X	X
Ausc. Pulm	X	X	X	X	X	X
Ausc. Card	X	X	X	X	X	X
Palp. Abdom.	X	X	X	X	X	X
Alt. MS	X	X	X	X	X	X
Alt. MI	X	X	X	X	X	X

Protocolo de seguimento – Análises/exames (SA – se aplicável)

	-15d	Baseline	1°Mês	2°Mês	3°Mês	6°Mês	9°Mês	12°Mês
DATA								
Hgb	X		X	X	X	X	X	X
Leucócitos	X		X	X	X	X	X	X
Neutróf.	X		X	X	X	X	X	X
Linfócitos	X		X	X	X	X	X	X
Plaquetas	X		X	X	X	X	X	X
VS	X		X	SA	X	X	SA	X
Tpo Protr.	X				SA	X	SA	X
INR	X				SA	X	SA	X
AST	X		X	X	X	X	X	X
ALT	X		X	X	X	X	X	X
Bil. Total	X		X	X	X	X	X	X
Bil. Dta	X		X	X	X	X	X	X
DHL	X		X	X	X	X	X	X
FA	X		X	X	X	X	X	X
GGT	X				X	X	X	X
Amilase	X				X	X	SA	X
Lipase	X				X	X	SA	X
Ureia	X		X	X	X	X	X	X
Creatinina	X		X	X	X	X	X	X
Ác. úrico	X					X		X
Proteínas Tot.	X					X		X
Albumina	X					X		X
Globulina	X					X		X
PCR	X		X	SA	X	X	X	X
Glicose	X				X	X		X
Col. Total	X				X	X		X
Col. HDL	X				X	X		X
Col. LDL	X				X	X		X
TGS	X				X	X		X
Lactato	X					X		X
Fósforo/Ca/VD	X					X		X
Anti-VHC	X							
RNAVHC		SA			SA	SA		SA
Genótipo		SA						
Atg. HBs	X							
AntiHBs	X							
AntiHBc	X							
AtgHBe	X							
AntiHBe	X							
DNAVHB		SA			SA			SA
Genótipo		SA						
AntiHAV IgG	X							
CV VIH	X			X	SA	X	SA	X
CD4	X		X	X	X	X	X	X
CD8	X		X	X	X	X	X	X

	16° Mês	20° Mês	24° Mês	28°Mês	32°Mês	36°Mês
DATA						
Hgb	X	X	X	X	X	X
Leucócitos	X	X	X	X	X	X
Neutróf.	X	X	X	X	X	X
Linfócitos	X	X	X	X	X	X
Plaquetas	X	X	X	X	X	X
VS	SA	SA	X	SA	SA	X
Tpo Protr.	SA		X		SA	X
INR	SA				SA	X
AST	X	X	X	X	X	X
ALT	X	X	X	X	X	X
Bil. Total	X	X	X	X	X	X
Bil. Dta	X	X	X	X	X	X
DHL	X	X	X	X	X	X
FA	X	X	X	X	X	X
GGT	SA	SA	X	SA	SA	X
Amilase	SA	SA	X	SA	SA	X
Lipase	SA	SA	X	SA	SA	X
Ureia	X	X	X	X	X	X
Creatinina	X	X	X	X	X	X
Ác. úrico	SA	SA	X	SA	SA	X
Proteínas Tot.	SA	SA	X	SA	SA	X
Albumina	SA	SA	X	SA	SA	X
Globulina	SA	SA	X	SA	SA	X
PCR	SA	SA	X	SA	SA	X
Glicose	SA	SA	X	SA	SA	X
Col. Total	SA	SA	X	SA	SA	X
Col. HDL	SA	SA	X	SA	SA	X
Col. LDL	SA	SA	X	SA	SA	X
TGS	SA	SA	X	SA	SA	X
Lactato	SA	SA	X	SA	SA	X
Fósforo	SA	SA	X	SA	SA	X
Anti-VHC			SA			SA
RNAVHC			SA			SA
Genótipo						
Atg. HBs						
AntiHBs						
AntiHBc						
AtgHBe						
AntiHBe						
DNAVHB			SA			SA
Genótipo						
AntiHAV IgG						
CV VIH	X	SA	X	SA	X	X
CD4	X	X	X	X	X	X
CD8	X	X	X	X	X	X

Protocolo de seguimento – Outras análises/exames

	-30d	Baseline	1°Mês	2°Mês	3°Mês	6°Mês	9°Mês	12°Mês
DATA								
VDRL	X				X			X
MHA-TP	X				X			X
Serol. VVZ	X							X
Serol. VHS1	X							X
Serol. VHS2	X							X
Serol. CMV	X							X
Serol. EBV	X							X
Serol. Toxopl.	X							X
LCR-células		X	SA	SA	SA	SA	SA	SA
LCR-proteínas		X	SA	SA	SA	SA	SA	SA
LCR-glicose		X	SA	SA	SA	SA	SA	SA
LCR-cript.(atgdc)		X	SA	SA	SA	SA	SA	SA
LCR-bact		X	SA	SA	SA	SA	SA	SA
LCR-BK		X	SA	SA	SA	SA	SA	SA
LCR PCR JC		X	SA	SA	SA	SA	SA	SA
LCR PCR EBV		X	SA	SA	SA	SA	SA	SA
LCR PCR CMV		X	SA	SA	SA	SA	SA	SA
LCR PCR Tox		X	SA	SA	SA	SA	SA	SA
LCR PCR BK		X	SA	SA	SA	SA	SA	SA
LCR PCR VIH		X	SA	SA	SA	SA	SA	SA
PCR/HC sg? HSV,BKv,PvB19 HTLV2			SA	SA	SA	SA	SA	SA
Mantoux	X	X(10-14d)			X			X
Quantiferon	X				X	SA		X
Rx	X				X			X
Ecografia Abd.	X					X		X
Fibroscan	X					X		X
TAC	SA	SA	SA	SA	SA	SA	SA	SA
RMN	SA	SA	SA	SA	SA	SA	SA	SA
TAC Timo	X							X
Outros: HLAB5701 Teste Resist. VIH Ex ginecológico Ex. oftalmol.(SA) Osteodensit. (SA) ECG (SA) UrinaII	X	SA UII	SA	SA	SA	SA UII	SA	SA UII

	16° Mês	20° Mês	24° Mês	28°Mês	32°Mês	36°Mês
DATA						
VDRL			SA			SA
MHA-TP			SA			SA
Serol. VVZ			SA			SA
Serol. VHS1			SA			SA
Serol. VHS2			SA			SA
Serol. CMV			SA			SA
Serol. EBV			SA			SA
Serol. Toxopl.			SA			SA
LCR-células	SA	SA	SA	SA	SA	SA
LCR-proteínas	SA	SA	SA	SA	SA	SA
LCR-glicose	SA	SA	SA	SA	SA	SA
LCR-cript.(atg,dc)	SA	SA	SA	SA	SA	SA
LCR-bact	SA	SA	SA	SA	SA	SA
LCR-BK	SA	SA	SA	SA	SA	SA
LCR PCR JC	SA	SA	SA	SA	SA	SA
LCR PCR EBV	SA	SA	SA	SA	SA	SA
LCR PCR CMV	SA	SA	SA	SA	SA	SA
LCR PCR Tox	SA	SA	SA	SA	SA	SA
LCR PCR BK	SA	SA	SA	SA	SA	SA
LCR PCR VIH	SA	SA	SA	SA	SA	SA
PCR/HC sg? HSV,BKv,PvB19 HTLV2	SA	SA	SA	SA	SA	SA
Mantoux			X			X
Quantiferon			X			X
Rx			X			X
Ecografia Abd.			X			X
Fibroscan			X			X
TAC	SA	SA	SA	SA	SA	SA
RMN	SA	SA	SA	SA	SA	SA
TAC Timo			X			X
Outros: Ex. oftalmológico Urina II SA: se aplicável	SA	SA	SA UII	SA	SA	SA UII

Infecções/patologias oportunistas após início da TARV:
Doenças AI:

Patologia
Data do início dos sintomas
Data do diagnóstico
Data do início do tratamento específico
Corticoterapia?

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_____
_____
_____
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_____
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