Universidade de Lisboa

Faculdade de Medicina

HIV-2 immunopathology: how the immune system deals with long-lasting infection

Rita Tendeiro

Doutoramento em Ciências Biomédicas
Especialidade de Imunologia

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Tese orientada pela Professora Doutora Ana E. Sousa

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Para a minha Mãe.

Para os meus avós, Guida e João.

To Freddie, you are my equal and my likeness.

"It takes all the running you can do,
to keep in the same place."

Lewis Carroll

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ABBREVIATIONS

A – adenine

Aa – amino acid

Ag – antigen

AGM – African green monkeys

AICD – activation-induced cell death

AIDS – Acquired Immunodeficiency Syndrome

Akt – protein kinase B

APC – allophycocyanin

APC – antigen-presenting cells

APOBEC – apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like

ART – antiretroviral therapy

AZT – 3'-azido-2',3'-dideoxythymidine

BAFF – B-cell activating factor

BST-2 – bone marrow stromal cell antigen 2

C – cytosine

CA – capsid protein

CD – cluster of differentiation

CDC – Centres for Disease Control and Prevention (USA)

CDK – cyclin-dependent kinase

cDNA – complementary DNA

CMV – cytomegalovirus

CO_2 – carbon dioxide

CRF – circulating recombinant form

cTEC – cortical thymic epithelial cells

DC – dendritic cells

DC-SIGN – dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin

DC-SIGNR – DC-SIGN related

DN – double-negative

DNA – deoxyribonucleic acid

DP – double-positive

EBV – Epstein-Barr virus

ELISA – enzyme-linked immunosorbent assay

Env – envelope glycoprotein

FDA – Food and Drug Administration (USA)

FDC – follicular dendritic cells

FISH – fluorescence *in situ* hybridization

FITC – fluorescein isothiocyanate

FoxP1 – forkhead box protein 1

FoxP3 – forkhead box protein 3

FRC – fibroblastic reticular cells

G – guanine

Gag – group-specific antigen

Gal-9 – galectin-9

GALT – gut-associated lymphoid tissue

Gp – glycoprotein

HIV – Human Immunodeficiency Virus

HMG-I(Y) – high motility group protein

HSC – hematopoietic stem cells

HTLV – Human T-Lymphotropic Virus

IDO – indoleamine 2,3-dyoxigenase

IFN – interferon

Ig – immunoglobulin

IL – interleukin

IL-7Rα – IL-7 receptor α chain (CD127)

IL-R – interleukin receptor

IN – integrase

ITIM – immunoreceptor tyrosine-based inhibitory motif

iTregs – induced Tregs

ITSM – immunoreceptor tyrosine-based switch motif

kb – kilobase

kDa – kiloDalton

LAV – Lymphadenopathy-Associated Virus

LBP – lipopolysaccharide-binding protein

LPS – lipopolysaccharide

LTR – long terminal repeat

LT-β – lymphotoxin-β

MA – matrix protein

mAb – monoclonal antibody

MCP-1 – monocyte chemoattractant protein-1

mDC – myeloid dendritic cells

MFI – mean/median fluorescence intensity (as specified)

MHC – major histocompatibility complex

MIP-1β – macrophage inflammatory protein-1β

MLR – multiple linear regression

MND – mandrills

mRNA – messenger RNA

mTEC – medullary thymic epithelial cells

mTOR – mammalian target of rapamycin

NC – nucleocapsid protein

Nef – negative factor

NF-κB – nuclear factor kappa B

NHP – non-human primate

NK – natural killer

NLS – nuclear localization signal

NNRTI – non-nucleoside analogs

NPC – nucleopore complex

NRTI – nucleoside analogs

NSI – non-syncytium inducing

nTreg – naturally occurring Treg

P6 – 6 kDa protein

PB – peripheral blood

PBMC – peripheral blood mononuclear cells

PBS – phosphate-buffered saline

PBS region – primer binding site (PBS) region

PCR – polymerase chain reaction

PD-1 – programmed-death protein 1 (CD279)

pDC – plasmacytoid dendritic cells

PD-L1 – programmed-death protein ligand 1 (B7-H1, CD274)

PD-L2 – programmed-death protein ligand 2 (B7-DC, CD273)

PE – phycoerythrin

PECAM-1 – platelet endothelial cell adhesion molecule-1 (CD31)

PEP – post-exposure prophylaxis

PerCP – peridinin chlorophyll protein

PI3K – phosphatidylinositol-3-kinase

PIC – pre-integration complex

Pol – polymerase

PPT – polypurine tract sequence

PR – protease

PrEP – pre-exposure prophylaxis

PTK7 – protein tyrosine kinase 7

Rev – regulator of expression of viral proteins

RNA – ribonucleic acid

RNase – ribonuclease

RRE – Rev-responsive element

RT – reverse transcriptase

RTC – reverse transcription complex

RTE – recent thymic emigrants

RT-PCR – real-time PCR

SAMHD1 – SAM domain HD domain-containing protein 1

SI – syncytium-inducing

SIDA – Síndrome da Imunodeficiência Adquirida

SIV – Simian Immunodeficiency Virus (SIVcpz, SIVsm)

sj – signal-joint

SLO – secondary lymphoid organs

SP – single-positive

STI – sexually transmitted infections

SU – surface glycoprotein

T – thymine

TAR – transactivation response region

Tat – transcriptional transactivator

TCR – T-cell receptor

TERC – telomerase RNA component

TERT – telomerase reverse transcriptase

TGF – tumor growth factor

Th – T helper

TIM-3 – T-cell immunoglobulin and mucin domain-containing molecule 3

TLR – Toll-like receptors

TM – transmembrane glycoprotein

TNF-α – tumor necrosis factor alpha

TREC – T-cell receptor excision circles

Treg – regulatory T-cell

TRIM – tripartite motif

tRNA – transfer RNA

Trp – tryptophan

Vif – viral infectivity factor

VIH – Vírus da Imunodeficiência Humana

Vpr – viral protein R

Vpu – viral protein U

Vpx – viral protein X

γc – common gamma chain (CD132)

SUMÁRIO

A infecção pelo Vírus da Imunodeficiência Humana (VIH) caracteriza-se por perda progressiva de células T $CD4^+$, conduzindo a Síndrome da Imunodeficiência Adquirida (SIDA). Actualmente, mais de 30 milhões de pessoas vivem com VIH. Portugal apresenta uma das incidências mais elevadas de infecção VIH-1 da Europa, e a prevalência mais alta de VIH-2, uma infecção essencialmente confinada à África Ocidental.

A infecção pelo VIH-2 constitui uma forma naturalmente atenuada de doença, dada a perda lenta de células T $CD4^+$, os níveis indetectáveis/baixos de vírus circulante e o impacto reduzido na mortalidade. Contudo, doentes VIH-2+ e VIH-1+ com igual número de células T $CD4^+$ apresentam números semelhantes de células infectadas e níveis comparáveis de hiper-activação imunitária, indicando que também o VIH-2 está associado a estimulação imunitária persistente.

Este trabalho baseou-se no pressuposto de que a investigação da infecção pelo VIH-2 revela estratégias usadas pelo sistema imunitário para enfrentar os desafios impostos pela infecção VIH crónica, elucidando a imunopatogénese associada ao VIH/SIDA.

A progressão lenta da infecção pelo VIH-2 relacionou-se com dois factores. Em primeiro lugar, a regulação estrita de vias inibitórias de activação celular, com sobre-expressão de moléculas inibitórias por células apresentadoras de antigénio e linfócitos T a constituir uma "assinatura" específica do VIH-2. Em segundo lugar, a preservação dos telómeros das células T $CD4^+$ e da capacidade de resposta das células T $CD4^+$ *naïve* à IL-7, contribuindo, provavelmente, para manter as células T $CD4^+$. Contudo, a perda de células B de memória, mesmo em doentes avirémicos, indica que, ao longo da fase "assintomática/crónica" mais prolongada na doença VIH-2, ocorrem alterações irreversíveis e, possivelmente, exaustão do sistema imunitário.

Este trabalho demonstra que a infecção pelo VIH-2 constitui um modelo natural único para identificar potenciais alvos para novas estratégias terapêuticas, complementares dos fármacos anti-retrovirais, que permitam uma efectiva reconstituição imunológica na infecção VIH/SIDA.

Palavras-Chave: Infecção VIH/SIDA, VIH-2, Activação Imunitária, Moléculas Inibitórias, Homeostasia das Células T $CD4^+$, Telómeros, Células T $CD4^+$ Naïve, IL-7, Células B de Memória.

SUMMARY

Human Immunodeficiency Virus (HIV) infection is characterized by progressive $CD4^+$ T-cell loss, ultimately leading to Acquired Immunodeficiency Syndrome (AIDS), and death. More than 30 million people live with HIV. In Western Europe, Portugal has one of the highest incidence rates of HIV-1 infection, and the highest prevalence of HIV-2 disease, which is otherwise mainly confined to West African countries.

HIV-2 infection, a naturally-occurring form of attenuated HIV disease, features a very slow rate of $CD4^+$ T-cell depletion, and undetectable to low levels of circulating virus, with limited impact in adult mortality. This is despite total numbers of infected cells being similar to those found in HIV-1 infection. Furthermore, HIV-2+ and HIV-1+ individuals with similar degrees of $CD4^+$ T-cell depletion have comparable levels of hyper-immune activation, indicating that persistent immune stimulation also occurs in HIV-2 disease.

This work aimed at investigating strategies mounted by the immune system in order to cope with challenges imposed by chronic HIV disease, using HIV-2 infection as an approach to study HIV/AIDS-related immunopathogenesis.

We identified two main factors possibly contributing to the slow HIV-2 disease progression. Firstly, a tight regulation of T-cell activation-inhibition pathways, with over-expression of inhibitory molecules on antigen-presenting and T cells constituting a specific “HIV-2 signature”. Secondly, a preservation of $CD4^+$ T-cell telomere length and sustained naïve $CD4^+$ T-cell ability to respond to IL-7, which likely impact on the capacity to replenish the $CD4^+$ T-cell compartment in HIV-2 infected individuals. Nevertheless, our finding of memory B-cell loss, even in aviremic HIV-2 disease, indicates that, over the prolonged “asymptomatic/chronic” phase of HIV-2 infection, irreversible damage and/or exhaustion of resources of the immune system ultimately occurs.

This study demonstrated that HIV-2 infection provides a unique natural model to identify rationales for novel therapeutic strategies complementary to antiretroviral drugs, in order to achieve immune-competence in HIV/AIDS.

Keywords: HIV/AIDS, HIV-2, Immune Activation, Inhibitory Molecules, $CD4^+$ T-Cell Homeostasis, Telomeres, Naïve $CD4^+$ T Cells, IL-7, Memory B cells.

CHAPTER 1: INTRODUCTION

The Human Immunodeficiency Virus (HIV) infection still represents one of the most important Global Health threats.

This chapter is divided into three parts: the first overviews HIV and the natural course of HIV disease, as well as the mechanisms underlying progression to Acquired Immunodeficiency Syndrome (AIDS); the second addresses persistent immune activation as the main determinant of HIV/AIDS immunopathogenesis; and, finally, the third part focuses on $CD4^+$ T-cell replenishment, a critical process to counteract $CD4^+$ T-cell loss, the hallmark of HIV/AIDS.

Part 1. The Human Immunodeficiency Virus and AIDS

When, in the early 1980's, severe cases of *Pneumocystis carinii* pneumonia were reported in previously healthy, young individuals, at three different hospitals in Los Angeles [1], the World in general, and the Scientific Community, in particular, were far from realizing that those were only the early days of a battle that would soon turn into the most challenging of wars.

Today, 30 years after AIDS was first recognized, and its causative agent, HIV, identified, the epidemic has spread to all nations, although varying in prevalence, with Sub-Saharan countries bearing an inordinate share of the global HIV burden (Figure 1). The pandemic seems to have stabilized in recent years, but it is still far from eradication: by the end of 2009, 33.3 million adults and children were living with HIV and, in that year alone, 2.6 million were newly infected, whilst 1.8 million lost their lives due to AIDS-related illness [2].

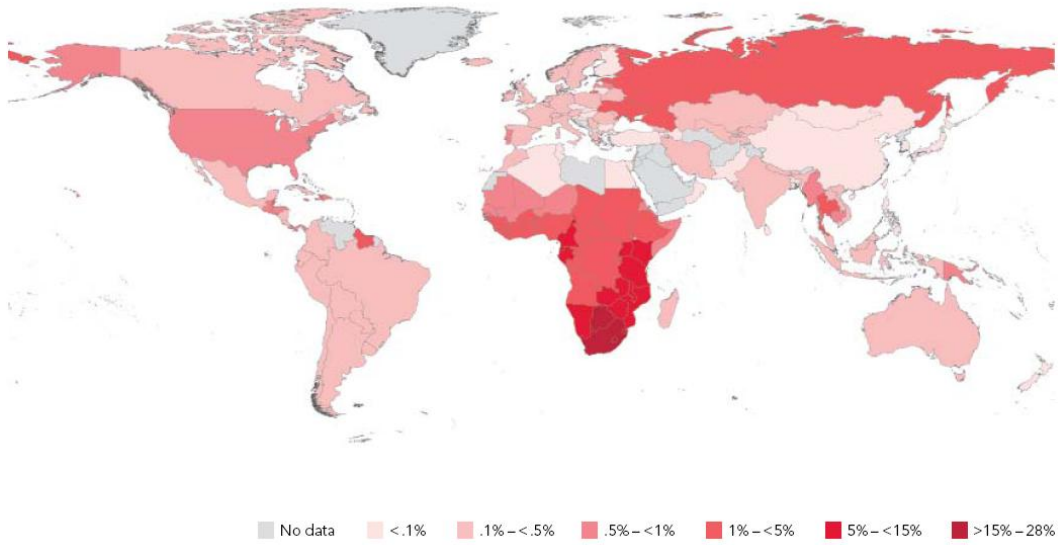

Figure 1. Global prevalence of HIV by the end of 2009. *UNAIDS report on the global AIDS epidemic, 2010.*

1.1. Identification, taxonomic classification and origin of the virus

The causative agent of AIDS was first isolated and identified in 1983, by Luc Montagnier and colleagues at the Institute Pasteur, France, who named it Lymphadenopathy-Associated Virus (LAV) [3]. At the same time, in the United Stated of America, Human T-Lymphotropic Virus (HTLV)-III was described by Robert Gallo as the virus causing AIDS disease [4]. The two original papers appeared back-to-back in *Science* and a legal dispute without precedent on who to give credit for the discovery was then initiated [5-7]. It was in this context that, in 1986, the International Committee on Taxonomy of Viruses, in order to standardize the nomenclature used in the increasing number of publications being added to the literature, whilst maintaining impartiality, ruled that both names should be dropped and replaced by a new one: Human Immunodeficiency Virus (HIV) [8].

Also in 1986, at the Institute Pasteur and in collaboration with Portuguese researchers, a second type of HIV was isolated from West-African patients [9], which was named HIV-2.

HIV belongs to the *Retroviridae* family, which includes all RNA reverse transcribing viruses, subfamily *Orthoretrovirinae*, genus *Lentivirus* [10]. Lentiviral (*lente*-, Latin for "slow") infections are characterized by long latency periods.

Both HIV-1 and HIV-2 are able to cause infection and disease in humans. However, while HIV-1 generated a worldwide disseminated pandemic, HIV-2 infection is much less prevalent and more geographically restricted, being primarily found in West African countries, India and, to a more limited extent, Portugal [11]. Of note, due to its historical and commercial links to West Africa, Portugal has been reported as the country in the European Union with the highest prevalence of HIV-2 infection [12-16] which, by the end of 2009, represented 3.2% of all reported AIDS cases since 1983 [17].

HIV-1 is thought to have arisen from cross-species (or zoonotic) transmission of the Simian Immunodeficiency Virus (SIV) from chimpanzees (SIVcpz) to humans [18], while HIV-2 is believed to have had its origin on the virus from sooty mangabeys (SIVsm) [19]. These zoonotic transmission events are thought to have been multiple and distinct (Figure 2), and are probably still ongoing.

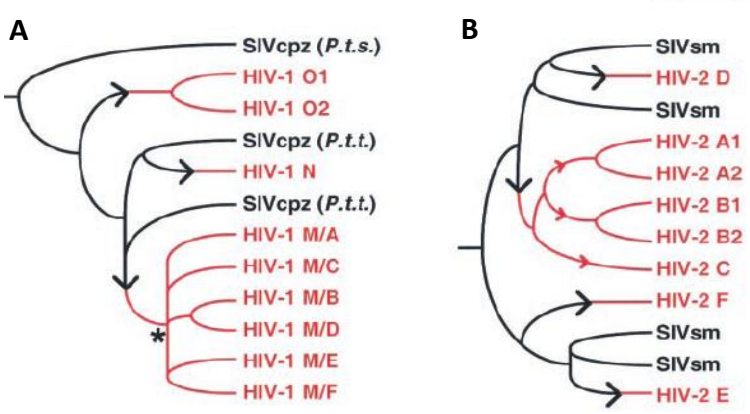

Figure 2. Multiple independent zoonotic transmissions of SIVcpz and SIVsm to humans. Black branches indicate evolution of SIV within natural hosts, black arrows indicate points of cross-species transmission, and red branches indicate subsequent evolution within human hosts. **(A)** SIVcpz and HIV-1. Groups M, N and O are interspersed among SIVcpz strains from *Pan troglodytes troglodytes* (*P.t.t.*) and *Pan troglodytes schweinfurthii* (*P.t.s.*). Six of the nine group M subtypes are depicted, all deriving from a common ancestor indicated by a black asterisk. **(B)** SIVsm and HIV-2. Six of the eight HIV-2 subtypes are represented, interspersed among SIVsm lineages. Adapted from *Hahn et al, Science 2000*.

As for HIV-1, at least three cross-species viral transmission events may have taken place from chimpanzees to humans [20, 21], giving rise to groups M (major/main) [22], O (outlier) [22, 23] and N (non-M, non-O) [24]; more recently, an additional one may have been responsible for generating the gorilla-derived group P [25]. Most HIV-1 infections are caused by group M viruses, which are divided into nine subtypes (A-D, F-H, J and K) [11].

Similarly, at least eight separate zoonotic transmission events [26] may have generated different HIV-2 subtypes (A-H) [27-30] in the human population. The majority of HIV-2 infections are caused by subtypes A or B, which have been referred to as the epidemic groups, while non-epidemic subtypes are known only as single-person infections and do not appear to be transmissible among humans [26, 30, 31].

Determining the timing of the virus introduction in the human population has been a priority for HIV researchers. Estimates based on viral genome sequences predict that SIV has given rise to transmissible HIV lineages throughout the twentieth century, with HIV-1 groups M, O and N having been introduced in about 1908, 1920 and 1963, respectively [26, 32, 33], and HIV-2 groups A and B appearing only in the early 1930s [26].

1.2. Structural biology and life cycle of the viral particle

1.2.1. Morphology and constitution

HIV virions are spherical in shape, with a diameter of, approximately, 1/10,000 of a millimeter (Figure 3). The outer coat of the virus, known as the viral envelope, consists of a lipid bilayer which derives from the infected cell's membrane during budding of new viral particles [34]. Embedded throughout the viral envelope are host cell's proteins [35], together with clusters of viral glycoprotein (gp) heterodimers – often referred to as spikes – with the function of mediating virion's attachment, docking and fusion with the host cell's membrane, hence determining viral tropism [36]. Viral glycoprotein heterodimers consist of two portions, each one with a distinct

molecular weight and designated accordingly: an outer glycoprotein (surface, SU) – gp120 in HIV-1, gp105 in HIV-2 – and a transmembrane (TM) glycoprotein – gp41 in HIV-1, gp36 in HIV-2. Each viral spike comprises three surface molecules – the "head" – noncovalently attached to three transmembrane domains – the "stalk" [36, 37].

The envelope's inner surface is lined with the matrix (MA) protein, a myristoylated protein of 17 kDa in HIV-1 (p17) and 16 kDa in HIV-2 (p16) [38], which is known to be crucial for virion assembly [39-41].

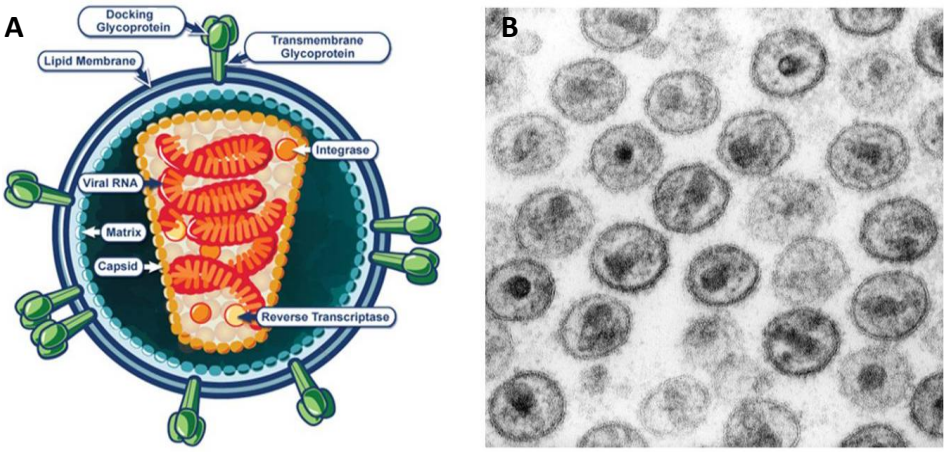

Figure 3. HIV virion. **(A)** Schematic representation of HIV's structure, with the identification of viral proteins and genome. Adapted from *www.niaid.nih.gov*. **(B)** Transmission electron photograph of HIV. Cone shaped cores are sectioned in various orientations. Viral genomic RNA is located in the electron-densewide end of core. Available at *phil.cdc.gov* (ID#948).

Protected by the viral envelope, the capsid, also called viral core, is a cone-shaped, highly organized multimeric structure [42], resulting from the assembly of multiple monomers of either a 24 or 26 kDa capsid protein (CA) in HIV-1 and HIV-2, respectively. The capsid contains the viral genome, which is associated with basic proteins of 7 and 6 kDa (nucleocapsid, NC, and p6, respectively), along with viral enzymes required for different replication events – reverse transcriptase (RT), integrase (IN) and protease (PR) –, some viral accessory and cellular proteins [42], and transfer RNAs (tRNAs), the latter of which serve as primers for reverse transcription [43]. The viral core's main function is to organize and contain the viral genome for optimal delivery and efficient reverse transcription in target cells which, together,

contribute to effective replication in the new host cell, a process absolutely required for infection [42].

1.2.2. Genome

HIV is a diploid virus, its genome consisting of two plus-stranded RNA copies. Each one of the RNA copies is about 9-10 kb long [44] and flanked by long terminal repeats (LTRs) that, although not encoding any proteins, are important for the regulation of viral transcription [34, 44]. Despite having similar genetic structures (Figure 4), HIV-1 and HIV-2 genome sequences may differ up to 60%, with HIV-2 genome being far more homolog to that of SIVsm (up to 77% homology) [44-46].

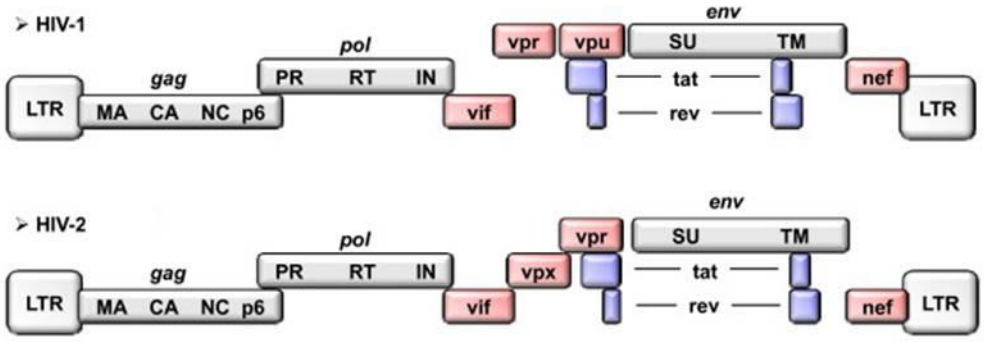

Figure 4. Schematic representation of HIV-1 and HIV-2 genomes. Grey boxes represent structural genes; blue boxes indicate regulatory genes; and pink boxes refer to accessory genes. Adapted from *Ayende et al, Retrovirology 2010*.

In addition to the genes common to all members of the *Retroviridae* family – *gag* (for "group-specific antigen", coding for the structural components NC, MA, CA, and p6), *pol* (for "polymerase", underlying the production of molecules with essential enzymatic functions: RT, PR, and IN) and *env* (for "envelope", coding for the surface and transmembrane glycoproteins of the viral envelope) –, HIV also features genes encoding a number of regulatory and accessory proteins that regulate different steps of viral replication and may influence pathogenesis [47, 48].

Viral proteins with regulatory functions (Tat – "transcriptional transactivator" – and Rev – "regulator of expression of viral proteins") are involved in promoting and

regulating viral transcription, being essential for effective viral replication, and will be discussed subsequently.

Accessory proteins are small molecules that play several roles during HIV's life cycle, deriving their name from the observation that, although being of utmost importance *in vivo*, they are not essential for *in vitro* viral replication in certain cellular systems, such as immortalized T-cell lines. Accessory proteins include Vif ("viral infectivity factor"), Vpu ("viral protein U", exclusive to HIV-1), Vpr ("viral protein R"), Vpx ("viral protein X", exclusive to HIV-2) and Nef ("negative factor"), with evidence suggesting that *vpx* may have arisen by duplication of an ancestral *vpr* gene within the HIV-2 lineage [49]. Among the many functions attributed to accessory proteins, some are of particular interest, such as: promotion of provirus formation and enhancement of progeny virions release by negative interference with host restriction factors (Vif [50] and Vpu [51]); transport of the pre-integration complex (PIC) into the nucleus and activation of LTR transcription (Vpr [52]); antagonism of a host restriction factor which counteracts the synthesis of proviral DNA in myeloid cells (Vpx [47, 53, 54]); and avoidance of super-infection and immune surveillance through down-regulation of both specific cellular receptors needed for viral entry and major histocompatibility complex (MHC) molecules, respectively (Nef [55]). Overall, HIV accessory proteins mainly constitute a means to neutralize specific host factors that would, otherwise, compromise the efficiency of viral replication.

1.2.3. Replication and assembly

HIV's life cycle may be divided into two distinct phases: an early phase referring to the initial steps of infection, from cell binding to the integration of proviral DNA into the host cell's genome; and a late phase beginning with the expression of viral genes and continuing up to the release and maturation of new progeny virions.

Figure 5 illustrates the different steps involved in this process, which is briefly addressed during the following sections.

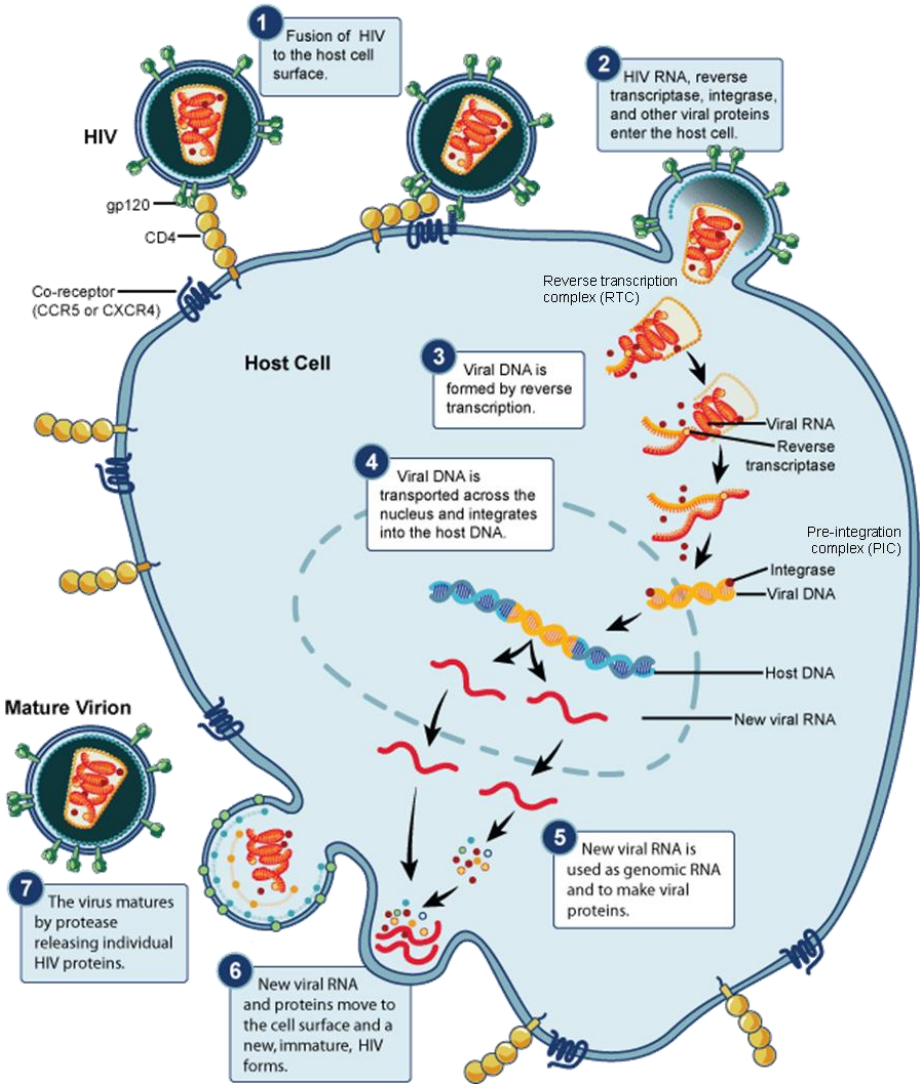

Figure 5. Schematic representation of HIV's life cycle. Adapted from *www.niaid.nih.gov*.

"Finding the key under the mat": the use of specific cell-surface (co)receptor(s)

The CD4 glycoprotein constitutes the high-affinity receptor for HIV [56], being mainly expressed on $CD4^+$ T cells, dendritic cells (DC) and monocytes/macrophages. Viral entry then typically depends on the sequential interaction of the viral envelope surface glycoproteins with this receptor and a seven-transmembrane-domain chemokine receptor [57] – the so-called coreceptor.

Although several chemokine receptors may function as HIV-1 coreceptors *in vitro* [58-60], multiple data suggest that only two of them, CCR5 and CXCR4, are of clinical relevance [61-64], with viral isolates being classified based on their ability to use CCR5 (non-syncytium inducing, NSI, R5 variants), CXCR4 (syncytium-inducing, SI, X4 variants), or both (R5X4 variants) as coreceptors for viral entry [57].

HIV-2 is able to bind a larger panel of cellular coreceptors [65-68], with some strains being capable of interacting with host cells even in the absence of CD4 [69]. Nevertheless, this HIV-2-associated increased promiscuity in terms of coreceptor usage has been shown not to be related with increased disease severity [70].

HIV-1, HIV-2 and SIV are also able to bind the surface of dendritic cells through interactions of their envelope glycoproteins with the C-type mannose binding lectins DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin) and DC-SIGNR (DC-SIGN related) [71-73]. However, these molecules should not be considered (co)receptors, as they do not promote viral entry and productive infection; rather, they may allow HIV particles to use DC as a Trojan horses, and thus be of more relevance to viral dissemination [74, 75].

Coreceptor usage has implications for viral tropism and is determined by the viral envelope [76]. The mechanisms of viral attachment and fusion with the target cell have been extensively described for HIV-1, being likely similar for HIV-2, given the similarities in structure and functional domains of Env glycoproteins observed in both virions [77]. The initial binding of gp120 to cellular CD4 triggers a conformational change in the surface glycoprotein, leading to the exposure of the coreceptor binding site [61, 78]. The interaction of CD4-bound gp120 with the coreceptor induces further conformational changes, leading to the exposure of the N-terminus of gp41, which contains a fusion peptide that embeds into the host cell's membrane [78]. The gp41 ectodomain forms an extended coiled-coil conformation and a six-helix bundle structure, which promotes the juxtaposition of the viral and target cell membranes [78]. This results in a stable structure, facilitating the fusion between viral and cellular membranes and the release of the viral core into the host cell's cytoplasm [58, 78].

"Unpacking to stay": reverse-transcription and pre-integration complexes

At this stage, the viral core undergoes destabilization and disassembly, also known as uncoating, leading to the generation of reverse transcription complexes (RTCs) and pre-integration complexes (PICs) [42]. RTCs are defined as HIV complexes that undergo reverse transcription (Figure 6), during which they convert their single-stranded positive RNA viral genome into double-stranded complementary DNA (cDNA); RTC genomes are, thus, either RNA or RNA-DNA intermediates of reverse transcription [73, 79]. In contrast, PICs no longer contain any RNA, but only the double-stranded cDNA, along with, at least, four viral proteins (MA, RT, IN and Vpr), representing, by definition, the integration-competent complexes [73, 79, 80].

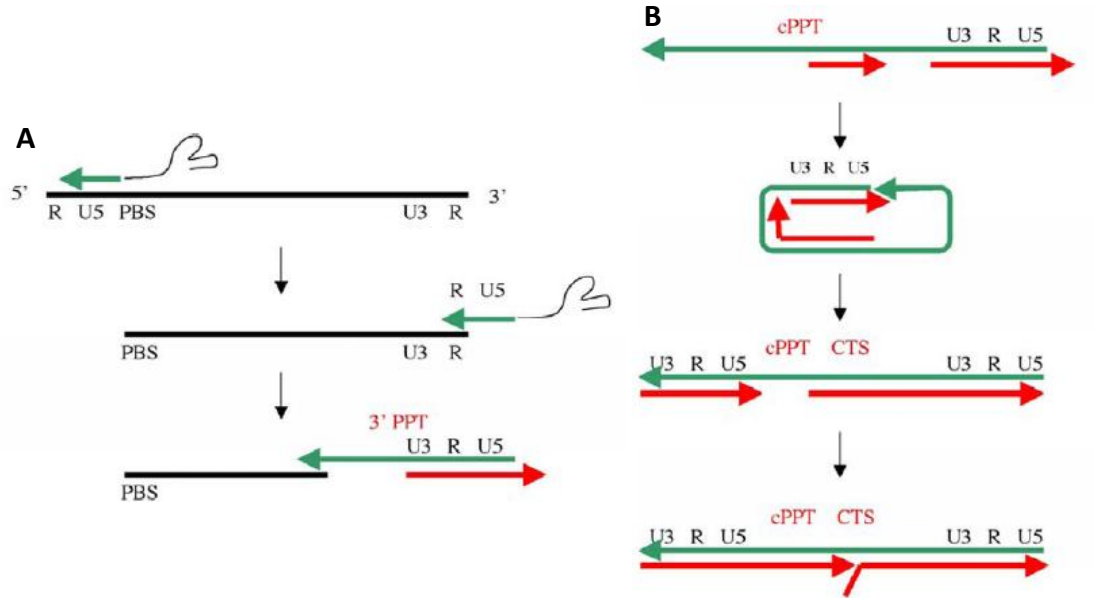

Figure 6. Schematic representation of the reverse transcription reaction, during which viral RNA is copied into a double-stranded cDNA molecule. The viral RT possesses three essential activities that are important for viral replication: RNA-dependent DNA polymerase (responsible for the process of reverse transcription), RNase H (mediating the cleavage of the genomic RNA in RNA/DNA hybrids during cDNA synthesis), and DNA-dependent DNA polymerase (for the synthesis of the second strand of the proviral DNA) [81]. **(A)** The process is mediated by the viral RT enzyme and a cellular tRNA3lys. The latter acts as primer and initiates negative strand cDNA synthesis, by binding to the primer binding site (PBS) region, located immediately 3' to the U5 region. During reverse transcription, the plus strand cDNA is synthesized discontinuously as two halves, the synthesis of one half being initiated from the 3' polypurine tract sequence (PPT), and the other **(B)** starting from the central copy of PPT (cPPT). Consequently, the final product is a linear cDNA molecule bearing in its centre a stable plus strand overlap, referred to as the central DNA flap. Adapted from *Arhel, Retrovirology 2010*.

The molecular mechanisms underlying HIV uncoating in the cytoplasm of infected cells remain to be elucidated. Although, in the beginning, the predominant

view was that the viral capsid was discarded immediately after fusion, close to the plasma membrane, in order to stimulate reverse transcription [79, 82-84], more recent data suggest that uncoating may, instead, be a gradual process [85]. This progressive uncoating would possibly take place in response to multiple cellular cues, such as interactions with cellular proteins or sub-cellular localization, or viral signals, such as the progress of reverse transcription. A yet third alternative suggests that viral capsids may remain intact until incoming complexes reach the nuclear membrane, with uncoating occurring at the nuclear pore upon completion of reverse transcription [42]. In any case, uncoating must be neither too early, nor too late, in order to productive infection to be ensured.

Despite the intricacies of HIV-1 uncoating being far from resolved, a model for the early steps of HIV infection has been recently proposed [42]: first, viral entry into target cells delivers the intact capsid core into the cytoplasm. Exposure of the viral nucleoprotein complex to non-limiting deoxyribonucleotides then triggers reverse transcription [73, 79], a process that likely occurs in the intact capsid core, which is essential for maintaining a high concentration of enzyme around the nucleic acid (that would, otherwise, detach [86]), while being entirely permeable to the necessary deoxyribonucleotides. During reverse transcription, RTCs move rapidly toward the nuclear compartment, using microtubules and actin filaments to reach the nuclear pore [87, 88]. Since transport to nuclear pore is faster than reverse transcription, it is likely that most viral cDNA synthesis occurs within capsids docked at the nuclear pore. Ultimately, capsid-free, double-stranded cDNA-containing, PICs enter the nucleus [89], leading to the integration of the DNA intermediate into the host cell's genome, where it is expressed under the control of host cell's transcription factors [80].

"Getting quietly settled": the establishment of a viral reservoir

Four different viral components have been identified that contribute to the nuclear import of HIV. The primary protein mediator of the PIC's nuclear import is IN. IN contains a short nuclear localization signal (NLS), a feature also shared by MA,

which is important for targeting the PIC to the nucleus [73, 79, 80]. Although lacking an NLS, Vpr has been shown to interact directly with components of the nucleopore complexes (NPCs), possibly enhancing nuclear import efficiency [90, 91]; however, the presence of this particular viral protein may not be strictly necessary, as Vpr-deficient viruses have been reported to maintain their ability to efficiently infect cells and cause pathology [92]. In contrast, the Vpx protein, encoded by HIV-2 and SIV, has been shown to be both necessary and sufficient for PIC's nuclear import [93]. Another component of the PIC that is important for nuclear entry is not a protein, but rather an unusual DNA structure; specifically, the DNA intermediate contains a discrete, centrally located region, where a third DNA strand forms a short overlap termed the central DNA flap [94]. This is formed during reverse transcription (Figure 6) and may enhance the nuclear entrance of PIC by recruiting cellular proteins that directly mediate its nuclear import. Alternatively, the DNA flap may be required for the PIC to achieve an appropriate conformation for import [88]. Of note, certain cellular proteins can also associate with the PIC during its journey to the nucleus, such as the high motility group protein HMG-I(Y), which has been proposed to be important for integration of the viral cDNA into the host cell's genome [95] to form the so-called provirus.

Proviral integration generally occurs in a non sequence-specific fashion, but may be influenced by the structure of the neighboring chromatin [96]. In agreement, HIV-1 integration has been described to preferentially occur in highly transcribed genes (sites of transcriptionally active chromatin) [97, 98], hence favoring efficient HIV-1 gene expression and maximizing virus propagation.

Viral cDNA integration is mediated by the virally-encoded and imported IN enzyme, in two sequential and distinct phases [99]: first, IN excises the two terminal nucleotides from each 3' terminus of the double-stranded viral cDNA (in a step called 3'-processing); 3'-processed viral cDNA is then covalently linked to host DNA in a process called joining, or strand transfer. Finally, single-stranded DNA breaks, at the site of integration in the host genome, are repaired by host factors, establishing the

integrated, latent, provirus, which will then remain permanently associated with the host's genetic material.

"Taking advantage of the situation": exploitation of the host cell's transcriptional and translational machinery

HIV proviral transcription is significantly influenced by the state of host cell activation and is regulated by sequences in the 5'-LTR of the viral genome, which are able to specifically bind numerous host cell transcription factors [100, 101]. In this way, HIV is able to harness the cellular transcriptional machinery in order to replicate, a feature resulting in the coordination of cellular activation and viral transcription.

The HIV 5'-LTR comprises three functionally discrete regions [102]: (1) the transactivation response region, TAR (which binds the virus-encoded trans-activating protein, Tat, and is essential to achieve significant levels of viral transcription); (2) the core promoter region (that mediates a basal level of viral transcription in response to SP1 transcription factors and TATA-binding proteins); and (3) the modulatory enhancer region, which contains several elements binding cellular transcription factors, including nuclear factor kappa B (NF-κB). On exposure of the cell to several activation stimuli, such as tumor necrosis factor alpha (TNF-α) [103], cytoplasmic NF-κB becomes physiologically active and translocates to the nucleus, where it mediates the activation of viral and cellular gene transcription by binding to the enhancer region [102]. Of note, while the HIV-1 5'-LTR presents a pair of NF-κB-binding DNA sequences in the enhancer region, HIV-2 has only one functional copy, and therefore responds more weakly to TNF-α stimulation [104, 105].

The basal transcriptional activity from the HIV LTR promoter is very low in most cell types, with very little messenger RNA (mRNA) being produced and translated into protein [106]; it can, however, increase significantly, leading to the production of large amounts of viral mRNA (and, consequently, protein) in activated cells. The switch from an inefficient to an efficient promoter is achieved, not only through the change in cellular factors, but also due to the action of Tat [107, 108].

Tat binds to the TAR sequence on the nascent mRNA molecule, recruiting the cyclin T1/CDK9 complex to this location, which then phosphorylates RNA polymerase II [109]. This leads to a dramatic stimulation of transcription, allowing for the synthesis of full-length mRNA transcripts to occur.

Transcription from the LTR generates a large number of viral mRNAs [110], which fall into three major classes [111]: 1) unspliced mRNAs (which function as the mRNAs for Gag and GagPol polyprotein precursors, and are packaged into progeny virions as genomic RNA); 2) incompletely spliced mRNAs (encoding the Env, Vif, Vpu, Vpr, and Vpx proteins); and 3) small, completely spliced mRNAs (which are translated into Tat, Rev and Nef) (Figure 7). Since most cellular mRNAs are fully spliced before their transport out of the nucleus, the need for unspliced and partially spliced RNAs to migrate to the cytoplasm poses a problem for HIV [111]. To circumvent this issue, the virus takes advantage of a system involving Rev and a specific RNA domain, the Rev-responsive element (RRE), that is located in the *env* gene and is present in all unspliced and partially spliced mRNAs [106, 112]. A Rev monomer initially binds to the RRE and, through protein-protein and RNA-protein interactions, multimerizes with other Rev molecules, eventually coating the RRE, and forming a complex that is capable of interacting with the cellular nuclear export machinery; as a consequence, the RRE-containing RNA is transported to the cytoplasm, after which Rev shuttles back to the nucleus, using its NLS [111, 112].

gag, *pol* and *env* are translated into Gag, Gag-Pol and Env polyprotein precursors [111]. Of note, while the genomic mRNA is translated by cytoplasmic ribosomes, *env* mRNA is translated on rough endoplasmic reticulum-associated ribosomes; then, the Env precursor enters the endoplasmic reticulum where it becomes glycosilated and is cleaved by cellular proteases into its SU and TM subunits [113]. The two glycoproteins remain associated and, through the Golgi, reach the plasma membrane, where they are either rapidly internalized (through recognition by the host cell machinery of an endocytosis motif) or incorporated into virus particles [114].

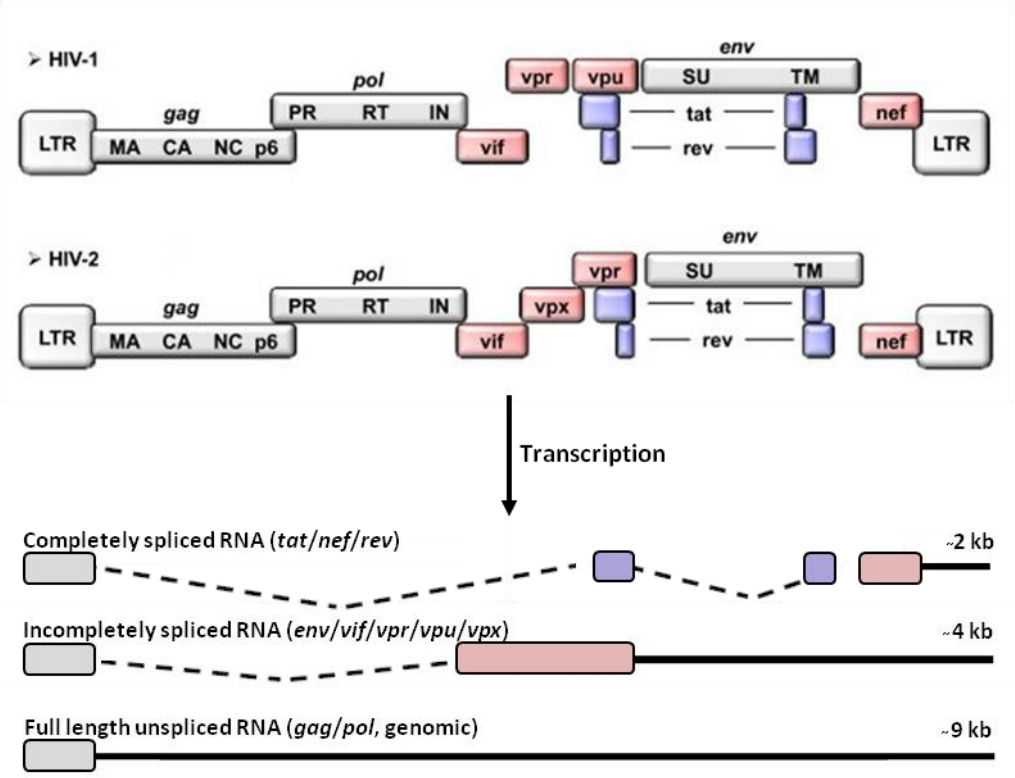

Figure 7. Schematic representation of mRNA species generated during HIV-1 and HIV-2 proviral DNA transcription. Grey boxes represent structural genes; blue boxes indicate regulatory genes; and pink boxes refer to accessory genes. Adapted from *Ayende et al, Retrovirology 2010* and *Nielsen et al, Retrovirology 2005*.

"The multiplying menace": viral progeny generation

New virus assembly begins at the plasma membrane. The major player in virus assembly is the Gag precursor polyprotein, which contains determinants that target it to the plasma membrane, bind the membrane itself, promote Gag-Gag interactions, encapsidate the viral RNA genome, associate with the viral Env glycoproteins, and stimulate budding from the host cell [111, 113, 115], with the consequent acquisition of the viral envelope.

During or shortly after virus release from the plasma membrane, the viral PR cleaves the Gag and Gag-Pol polyprotein precursors, generating the mature *gag*- and *pol*-encoded proteins, a process that leads to a series of structural rearrangements, ultimately resulting in virion maturation [111]. Importantly, this PR-mediated virion maturation has been described as essential for the production of competent viral progeny [116].

Of note, in the process of virus assembly, some host factors are also packaged in the new viral particles, including apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like (APOBEC) 3G and cyclophilin A [42, 111], the latter appearing to be HIV-1-specific [117, 118] and serving as a chaperone to prevent unfavorable CA aggregation [119].

"The virus with multiple faces": quasispecies and circulating recombinant forms

During HIV's life cycle, significant genetic variability is introduced. Both recombination and point mutations contribute to this variation, with base-substitutions being inserted mainly by error-prone DNA synthesis [120], or by the activity of cellular proteins, such as APOBEC3 cytidine deaminases [121, 122]. Of note, HIV recombination does not involve nucleic acid breakage and rejoining but, instead, results from RT template-switching between the two viral RNA molecules during provirus synthesis [123, 124] and lack of proofreading function [124]. As a result, in a given host, HIV exists as an array of quasispecies, in which nearly every virus in a generation differs from every other one.

Many of these viral genome recombination events result in the intertwining of genetic segments derived from different established subtypes (see Chapter 1.1.). Because some of these recombinants are able to spread among individuals, they have been called circulating recombinant forms (CRFs) [123]. There are currently 54 HIV-1 and one HIV-2 CRFs [125].

1.3. Natural history of HIV infection

Most HIV infections occur by sexual transmission, following exposure to cell-free or cell-associated virus in semen, vaginal secretions or mucosal surfaces [126]. Other routes of infection include transmission *via* intra-venous drug use, exposure to blood and blood products by transfusions, and exposure of fetus or infant to HIV from an

infected mother [126] (*in utero*, during delivery, or after birth, through breastfeeding). Of note, HIV-2 has lower progeny virus production and low viral load in body fluids than HIV-1, features that may account for the lower transmission rate observed for this infection [12]. For instance, sexual and mother-to-child transmissions of HIV-2 are around 5- to 9-fold and 10- to 20-fold reduced in relation to HIV-1, respectively [127].

"Playing hide-and-seek": acute/primary infection

Following transmission of HIV-1, there is a period of, approximately, ten days, known as the eclipse phase, before viral RNA becomes detectable in the plasma [128] (Figure 8). Evidence suggest that infection is initiated by a single virus, the so called "founder virus", indicating that a selection bottleneck may exist, leading to the preferential transmission of particular quasispecies [129-131].

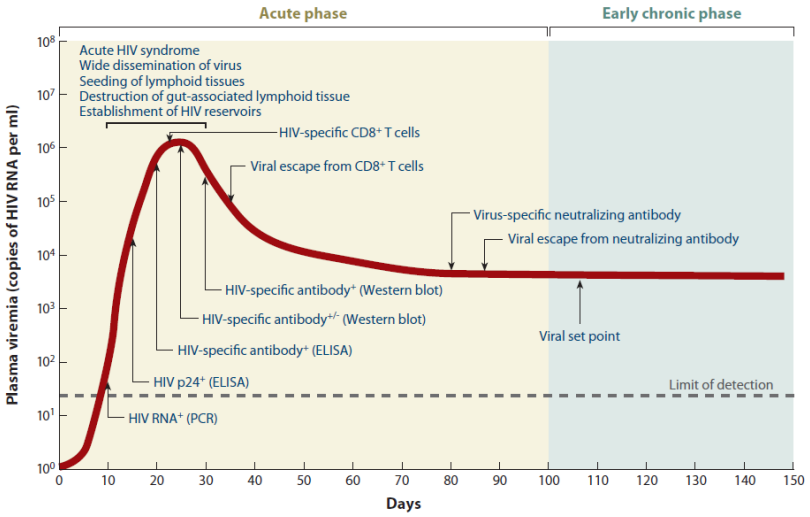

Figure 8. Kinetics of immunologic and virologic events observed during HIV acute/primary and early asymptomatic chronic phases. Approximate timings for positivity to HIV screening tests are shown. ELISA: enzyme-linked immunosorbent assay; PCR: polymerase chain reaction. *Moir et al, Annual Review of Pathology 2011*.

When the virus and/or virus-infected cells reach the draining lymph nodes, they encounter activated $CD4^+CCR5^+$ T cells, which constitute the ideal targets for further infection, due to the close association between cell activation and viral replication [102]. This process is enhanced by both DC and B cells that bind and carry the virus to activated T cells (*via* DC-SIGN [132] and CD21 [133], respectively). The virus then

replicates rapidly in the absence of an efficient immune response, spreading throughout the body to other lymphoid tissues, particularly the gut-associated lymphoid tissue (GALT), where activated $CD4^+CCR5^+$ memory T cells are present in high numbers [134]. This leads both to an exponential increase of plasma viremia, reaching a peak three to four weeks post-virus exposure, and to massive $CD4^+$ T-cell depletion in the gut [128, 135], which may be in turn related to the marked decrease observed in circulating $CD4^+$ T lymphocytes. Rapid viral replication and peak viremia may be associated, in HIV-1-infected individuals, with an acute clinical syndrome, defined as flu-like clinical manifestations [126]. No cases of acute HIV syndrome have been reported for HIV-2 infection.

Viral reservoirs are progressively established and may be divided into two main categories [126]: 1) cellular reservoirs consisting of mainly $CD4^+$ T cells (that are highly susceptible to viral replication upon activation, but also carry the virus in a latent proviral form), as well as monocytes and dendritic cells [136-139]; and 2) virus entrapped by follicular dendritic cells (FDC) in germinal centres within lymphoid tissues, mostly in immune complexes [140, 141].

After the point of peak viremia, viral load starts to progressively decrease, reaching a more stable level, known as the viral set point [126, 128, 142], process that occurs simultaneously with $CD4^+$ T-cell rebound. Virus diversification occurs during this decline in viral load, with multiple escape mutants being selected under the pressure of adaptive immune responses – both early HIV-specific $CD8^+$ T-cell responses, first detected just before peak viremia and possibly contributing to the decline in plasma viral load, and neutralizing antibodies [126, 128]. This viral set point, maintained by a balance between virus replication and the immune responses, is typically significantly higher for HIV-1 than for HIV-2-infected individuals [143].

"Forgotten but not gone": asymptomatic chronic infection

Following the primary phase of HIV infection, an asymptomatic period takes place, during which progressive $CD4^+$ T-cell depletion, ongoing viral replication and

overall immunological dysregulation still occur, albeit no or few clinical manifestations of the disease are observed [144, 145] (Figure 9A).

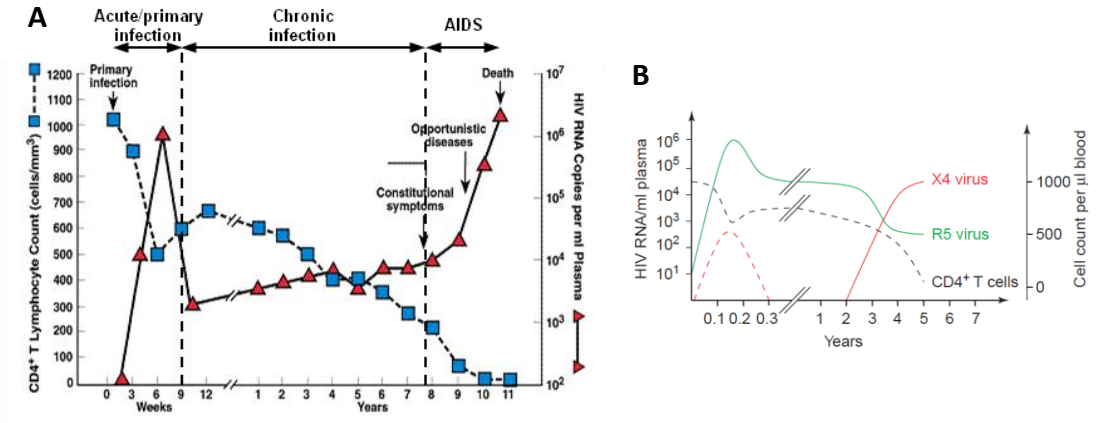

Figure 9. Natural history of HIV infection. **(A)** Clinical, immunological and virological evolution of HIV infection. Adapted from *Fauci et al*, *Annals of Internal Medicine 1996*. **(B)** Kinetics of CCR5- and CXCR4-using viral strains during the course of HIV infection. *Regoes&Bonhoeffer, Trends in Microbiology 2006*.

The length of asymptomatic chronic infection varies from patient to patient, and depends on a fine balance between viral replication and the ability to maintain immune resources. Typically, the rates of $CD4^+$ T-cell depletion are much more pronounced in HIV-1- than HIV-2-infected individuals, with plasma viral load remaining one to two $\log_{10}$ higher in the former, translating into a longer asymptomatic phase in HIV-2 infection [146].

Importantly, $CD4^+$ T-cell depletion during chronic HIV infection has been shown to be intricately related to hyper immune activation [144], which, as shall be discussed in detail later, constitutes a hallmark of HIV disease, playing an important role in the associated immunopathogenesis [102].

"The darkest hour": late infection and AIDS

As a consequence of the continuous viral replication and eventual collapse of the host's immune system, the final stage of HIV infection, AIDS, is set, ultimately leading to death (Figure 9A). The clinical state of AIDS may be defined, either by the occurrence of particular opportunistic infections [147], or on the basis of $CD4^+$ T-cell counts, with levels below 200 $CD4^+$ T cells/µl of blood being currently used as a

general AIDS-defining criteria implemented by the USA Centres for Disease Control and Prevention (CDC) [148].

"Changing keys": coreceptor switching and HIV disease progression

While CCR5-using viral strains seem to be the most commonly used for the initial establishment of HIV infection, predominating during the long asymptomatic phase, disease progression is often associated with the emergence of syncytium-inducing, CXCR4-using viral variants [61, 78, 149] (Figure 9B). Although the mechanisms driving coreceptor switching are still a matter of debate, this process appears to be important for HIV-1 pathogenesis, as individuals in whom CXCR4-using variants emerge (approximately 40-50% of all infected patients) display a more rapid decline in $CD4^+$ T cells and a significantly faster progression to AIDS and death than individuals in which viral variants only use CCR5 [58, 149]. This may be related with the expression of CXCR4 on a broader range of cells, including naïve $CD4^+$ T cells and thymic precursors, in comparison with the more restricted expression of CCR5 on activated memory $CD4^+$ T lymphocytes [61, 78].

Of note, previous data from our Laboratory have shown that the frequency of $CCR5^+$ cells within total memory $CD4^+$ T cells tended to be higher in HIV-2- than in HIV-1-infected individuals with the same degree of $CD4^+$ T-cell depletion, suggesting that the slower HIV-2 disease progression is not related with lack of CCR5 availability [150].

"Fighting fire with fire": host hindrance to HIV infection

HIV/AIDS pathogenesis involves complex interactions mediated by both viral and host-derived factors, depending on host genomics and immunological responses. Genetic determinants of host susceptibility to viral infection and replication include genes coding for chemokine receptors, as well as the so-called "host restriction factors": components of the innate immune responses that interfere with different

stages of the virus life cycle, being either constitutively expressed, or induced by interferon (IFN)-α production in response to viral infection.

Perhaps the most important example of chemokine receptor-mediated host resistance to infection is the naturally-occurring 32 base-pair deletion in the CCR5 gene (*CCR5Δ32*) resulting in a truncated protein product [151]. In the homozygous state, this deletion provides near complete protection from HIV infection [151, 152]; while in the heterozygous form it may confer a delay of up to 2-4 years in disease progression [153]. The CCR5Δ32 deletion is found at a frequency of 10-16% in Northern Europe, decreasing in a Southeast cline toward Mediterranean, and gradually disappearing in the African and Asian populations [154].

There are three main classes of restriction factors that are effective against HIV: tripartite motif (TRIM) proteins, cytidine deaminases, such as APOBECs, and tetherin.

TRIM5α is an interferon-induced [155, 156] antiviral factor that protects humans and other primates against a broad range of retroviruses, in a species-specific manner, by targeting the capsid molecules of the incoming virus in the cytoplasm and disrupting its replication [154]. The mechanism by which TRIM5α renders the virus uninfectious remains unclear, but it may be that, by coating the viral core with multivalent complexes, TRIM5α trimers are able to interfere with the uncoating process [157-159].

APOBECs are cytidine deaminases that induce G-to-A hypermutation in newly synthesized retroviral cDNA, resulting in instability of the nascent viral retrotranscripts or lethal mutations [160-162]. Although APOBEC3G was the first to be identified as an important antiretroviral factor [163], displaying the most potent anti-HIV-1 activity [164], other family members, such as APOBEC3F, 3H and 3B have also been shown to inhibit HIV-1 replication [165]. In non-activated $CD4^+$ T cells, APOBEC3G remains as an enzymatically active low-molecular mass form that hinders the accumulation of reverse transcripts upon viral cellular entry; after T-cell activation, the low-molecular mass complexes assemble into high molecular mass complexes without cytidine deaminase activity. This suggests that active APOBEC3G would represent a restriction

factor for the progress of infection in resting $CD4^+$ T cells, whereas in activated lymphocytes, inactive APOBEC3G would allow efficient retrotranscription of viral RNA [166, 167]. Of note, APOBEC3G is counteracted by the viral protein Vif [162], which neutralizes its action by three major mechanisms [165]: mediating its targeting for degradation, impairing the translation of its mRNA and preventing its incorporation into viral particles.

Tetherin (or CD317, or Bone Marrow Stromal Cell Antigen 2, BST-2) is a heavily glycosylated integral membrane protein that is usually only expressed efficiently on plasmacytoid dendritic cells (pDC), some cancer cells, terminally differentiated B cells and bone marrow stromal cells; however, its expression is strongly induced by type I IFNs [168]. With an unusual structure [169], it acts like a cross linker between the virions and the cellular membrane. This physical bond keeps the new viral particles attached to the plasma membrane, or to each other, impeding their release [170, 171]. The tetherin-mediated restriction of HIV-1 particle is only completely successful in the absence of Vpu, which is able to counteract its effects, either by targeting it to degradation [165], or by binding and redirecting it to perinuclear compartments [51, 172]. Of note, in HIV-2, where there is no Vpu, tetherin-mediated restriction is counteracted by Env [173-177].

A particular case of host resistance to productive viral infection is that observed in cells from the myeloid-lineage, including monocytes, dendritic cells and macrophages. Although these cells play an important role in initial HIV infection and viral dissemination, they seem to be restrictive to post-entry HIV-1 infection *in vitro* [136, 138, 178]. Nevertheless, the Vpx protein from SIVsm and HIV-2 lineages has been shown to be able to efficiently enhance HIV-1 infection in human DC and macrophages, promoting the accumulation of full-length viral cDNA [47, 179]. Recently, it has been shown that Vpx counteracts the effect of a particular host restriction factor – SAM domain HD domain-containing protein 1, SAMHD1 [180] – by mediating its degradation [53]. SAMHD1 was shown to be critical for HIV-1 replication restriction in non-permissive cells, including monocytes, monocyte-derived DC and macrophages [181], with SAMHD1-defficient monocytes being susceptible to HIV-1

infection [182]. Although many aspects of the biological function of SAMHD1 are still unknown, it has recently been proposed that SAMHD1 restricts HIV-1 replication by inhibiting reverse transcription and viral cDNA synthesis [183, 184]. SAMHD1-mediated HIV-1 restriction in myeloid-lineage cells, besides protecting them from efficient HIV-1 infection, has been suggested to impair their ability to present HIV antigens, thereby limiting the triggering of anti-HIV-1 specific T-cell responses [181]. Conversely, SIVsm and HIV-2 encode Vpx to overcome SAMHD1-mediated restriction, which probably induces antiviral immunity, thus limiting viral infection in natural hosts [181]. Accordingly, the interactions between SAMHD1 and Vpx may contribute to the differing consequences of HIV-1 and HIV-2 infections in humans.

1.4. Models of HIV/AIDS pathogenesis

1.4.1. Non-pathogenic vs. pathogenic SIV infection

SIV-infected non-human primates (NHPs), such as sooty mangabeys (SM), African green monkeys (AGM), mandrills (MND), and chimpanzees (CPZ), typically represent natural hosts of the virus, with no progression to disease being observed [185]. Less frequently, however, infection of NHP with SIV strains from other species results in pathology [21]. This is the case of the infection of Asian rhesus macaques (RM) with SIV_{SM}, which has been widely used as the model most closely resembling HIV-induced disease in humans [186-188]. Both non-pathogenic and pathogenic SIV infections have been extensively explored to investigate HIV/AIDS pathogenesis.

Most infected natural hosts are able to live a normal life, both in the wild and in captivity [189]. Only two cases of AIDS have been reported in animals that had been infected for very long periods of time [190, 191]. The exact mechanisms that make SIV infection generally non-pathogenic in natural NHP hosts remain elusive. Although these animals present levels of circulating virus that are remarkably similar to those found during pathogenic SIV/HIV infections, they tend to maintain preserved peripheral $CD4^+$ T-cell counts and reduced levels of generalized immune activation [192, 193] (Figure 10) which, as shall be discussed in detail later, is one of the

hallmarks of SIV/HIV disease [192, 194]. Interestingly, rare cases of $CD4^+$ T-cell depletion in the presence of low levels of immune activation have been reported in SIV natural hosts, with no progression to AIDS being observed [195], suggesting a protective role for reduced immune activation in non-pathogenic SIV disease, in particular, and HIV/AIDS pathogenesis, in general.

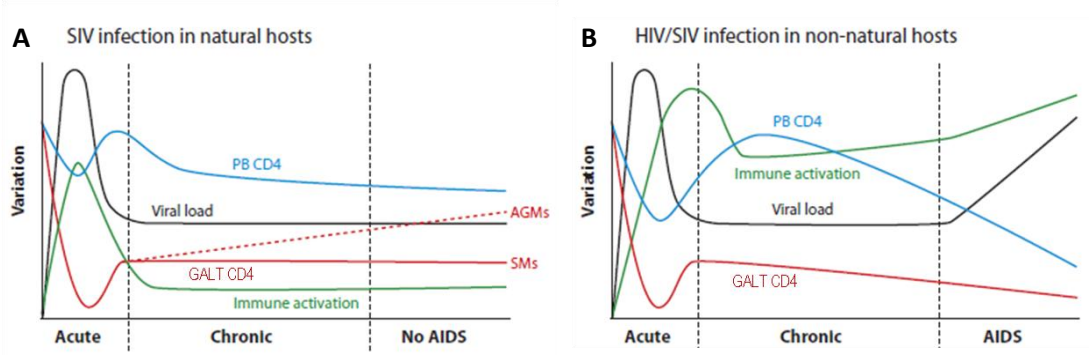

Figure 10. Non-pathogenic SIV *vs.* pathogenic SIV/HIV infections. Graphs illustrate the dynamics of viral load, $CD4^+$ T cells in peripheral blood (PB) and gut-associated lymphoid tissue (GALT), and immune activation during the course of **(A)** non-pathogenic SIV infection in natural hosts and **(B)** pathogenic SIV/HIV infections. SM: sooty mangabeys; AGM: African green monkeys. Adapted from *Paiardini et al, Annual Reviews of Medicine 2009*.

Of note, even though GALT $CD4^+$ T-cell depletion is a common feature of both pathogenic and non-pathogenic acute infection, its extent seems to be less dramatic in natural hosts than in progressive SIV/HIV diseases [192, 193]. Indeed, whereas depletion of mucosal $CD4^+$ T cells increases with HIV/SIV disease progression [135], in natural hosts the levels of mucosal $CD4^+$ T lymphocytes remain stable (SM), or even increase (AGM) during the chronic phase of infection [196]. The preservation of mucosal immunity in SIV-infected SM and AGM is in agreement with the observation that these animals do not show microbial translocation from the intestinal lumen to the systemic circulation, in contrast to what has been reported for pathogenic HIV/SIV infections [197-199].

Another key feature of natural SIV hosts is their paucity of CCR5 expression on $CD4^+$ T cells from blood, lymph nodes, and mucosal tissues [200], a phenotype that may be the result of a convergent evolutionary co-adaptation between primate

lentiviruses and their natural hosts, contributing to their non-pathogenic infection [192, 200].

1.4.2. Clinical models of HIV-1 infection

A minority of all HIV-1-infected patients manifest a non-progressive disease, and thus are of particular interest to investigate the mechanisms underlying natural control of HIV/AIDS immunopathology. These so-called "long-term non-progressors" (LTNP) were first described in the 1990s, and are characterized by their ability to naturally preserve $CD4^+$ T-cell counts above 500/µl of blood for, at least, ten years after infection [201-203].

Individuals who are able to maintain circulating viral load below the detection limits (less than 1% of all HIV-1-infected individuals) were subsequently identified, being referred to as "elite controllers" [204, 205]. A large proportion of "elite controllers" is thought to also meet the criteria for LTNP, though a slow progressive $CD4^+$ T-cell decline has been reported [206].

Both host and viral factors seem to be involved in this natural ability to control HIV-1 infection, in particular: the high prevalence of AIDS-protective HLA-B*27 and HLA-B*57 alleles [205, 207-209]; and the occurrence of *nef*-deleted HIV-1 mutants [210].

1.4.3. HIV-2 infection

The clinical spectrum of HIV-2 infection is similar to that observed for HIV-1 [211]. However, HIV-2 is generally less pathogenic, with most patients presenting a much slower disease progression, characterized by a clinically latent period of twenty or more years, that results in a mortality rate estimated to be two-thirds lower than the one observed for HIV-1 [212-214]. HIV-2 infection therefore constitutes a naturally-occurring attenuated form of HIV disease, providing an important

opportunity to study immune control and pathogenesis of HIV/AIDS that has been yet underestimated.

The mechanisms underlying the better disease prognosis associated with HIV-2 infection remain unclear. HIV-2-infected patients present a much slower rate of $CD4^+$ T-cell decline than their HIV-1+ counterparts [146] and low to undetectable circulating viral load [143]. Nevertheless, levels of proviral DNA have been reported to be similar in both infections [215, 216], with ongoing viral replication being observed even in aviremic HIV-2-infected individuals [216]. These findings suggest that, in the context of HIV-2 disease, productive viral infection and replication do occur but, nonetheless, fail to be translated into circulating virus. Of note, when HIV-1- and HIV-2-infected patients are matched for the degree of $CD4^+$ T-cell depletion, their levels of immune activation are similar [217], stressing the importance of this particular factor for HIV/AIDS immunopathology and associated disease progression.

1.5. Strategies for HIV control

1.5.1. Antiretroviral therapy (ART)

The advent of antiretroviral therapy has brought about a substantial decrease in the death rate associated with HIV-1 infection, changing it from a rapidly lethal disease into a chronic manageable condition, compatible with a very long survival of infected individuals [218].

Since the USA Food and Drug Administration (FDA) approval of 3'-azido-2',3'-dideoxythymidine (AZT), the first anti-HIV drug, in 1987, a variety of new therapies have arisen. There are now over 30 antiretroviral products, formulated either singly or in combination, to treat HIV-1-infected individuals [219]. These are subdivided into different classes of drugs based on the particular phases of the viral life-cycle they have been designed to target: fusion inhibitors, entry inhibitors (CCR5 antagonists), RT inhibitors (either nucleoside analogs, NRTI, or non-nucleoside analogs, NNRTI), integrase strand transfer inhibitors and protease inhibitors [218].

Despite the enormous success of antiretroviral drugs in hampering viral replication and reducing circulating viral load, disease progression and transmission, the cure of HIV/AIDS disease and the control of HIV reservoirs have not been achieved. In fact, once initiated, ART has to be maintained for life, with the inherent side-effects and economic burden [220]. The longer the period on ART, the higher the risk for emergence of drug-resistance and viral genetic diversity which, ultimately, lead to ART failure [221]. Thus, the definition of the best moment to start ART is critical, being the subject of an intense ongoing debate [221-223].

Regarding HIV-2 infection, ART regimens are associated with a much more reduced clinical success [146, 216, 224, 225]. Many factors are likely to contribute, including the fact that all antiretroviral drugs have been specifically designed to control HIV-1 replication. Apart from having an innate resistance to NNRTI [226, 227], HIV-2 may have decreased susceptibility to fusion inhibitors [223]. Of note, there are no randomized clinical trials for the treatment of HIV-2 infection, which complicates clinical decisions during the follow-up of HIV-2-infected individuals [223].

1.5.2. Cytokine-based complementary therapeutic interventions

ART alone is not sufficient to recover $CD4^+$ T-cell counts in about 20% of HIV-1-infected patients despite the efficient suppression of plasma viral load [228]. These so-called immunologic discordants highlighted the need for other strategies to be developed and used in conjunction to ART, in order to achieve full immunological reconstitution under treatment.

Due to their ability to induce both proliferation and survival, cytokines of the common γ-chain cytokine family – such as interleukins-2 (IL-2), -7 (IL-7), -15 (IL-15) and -21 (IL-21) – have emerged as possible central regulators of peripheral $CD4^+$ T-cell homeostasis during HIV infection [229]. Studies are ongoing to define the applicability of therapies based on the use of these cytokines as a complement to ART [230-237]. Whilst IL-2 appears to have a negligible impact on $CD4^+$ T-cell recovery in HIV-1-infected individuals on ART [238], the effects of IL-15 administration, besides

promoting extensive memory T-cell expansion in NHP [239], remain largely unknown [240, 241]. On the other hand, IL-7-based immunotherapy has recently been shown to enhance T-cell recovery and thymic output in HIV-1-infected patients on ART [242], rendering this particular cytokine an attractive therapeutic adjuvant in HIV disease.

Of note, as HIV is known to be able to persist in viral reservoirs that are not eliminated by ART [243], the use of proliferation-inducing common γ-chain cytokines, in particular IL-7, is thought to be of relevance to help "purge" the pool of latently infected cells, consequently contributing to viral eradication [233, 244, 245].

1.5.3. Preventive strategies

Besides treating HIV/AIDS disease, reducing the incidence of new infections currently remains one of the greatest Public Health challenges. No single prevention strategy is currently totally effective and applicable to all populations [246].

Male condoms still appear to constitute the most highly effective form of HIV prevention [247]. Other interventions, including the treatment of sexually transmitted infections (STI) [248], male circumcision [249, 250], and the use of non-antiretroviral microbicide vaginal gels [251], have been shown to be clinically successful in reducing HIV acquisition.

Recently, ART-based pre-exposure prophylaxis (PrEP) has also been proposed as a preventive strategy [252-254]. Preventive strategies relying on ART administration have been extensively used to avoid infection as post-exposure prophylaxis (PEP) after contact with HIV-infected blood or fluids [255, 256]. Although an intense public debate is still ongoing about the applicability of PrEP, the FDA has very recently approved its use for prevention in populations at high risk of sexually acquired HIV infection [257].

Part 2. Hyper immune activation as the main cause of $CD4^+$ T-cell depletion in HIV infection

Factors other than the direct killing of $CD4^+$ T cells by the virus have been suggested to be involved in HIV/AIDS immunopathology, in particular the persistently increased immune activation. In fact, during chronic HIV infection, the frequency of infected $CD4^+$ T lymphocytes has been shown to be too low to solely account for the observed $CD4^+$ T-cell loss [135, 258].

The idea that chronic immune activation could play a major role in AIDS pathogenesis was first proposed in the late 1980s/early 1990s [259-261]. Shortly after, data from several groups started to progressively accumulate, suggesting that levels of immune activation, particularly at the $CD8^+$ T-cell level [262], constituted a better correlate of disease progression than actual viral load, both in HIV-1 [217, 262-265], and HIV-2 [217] infections. Additionally, studies of SIV infection of natural hosts showed that, despite the high levels of circulating virus, there was no progression to AIDS in the absence of increased levels of immune activation [195, 266-268]. Taken together, these findings supported a new paradigm in HIV/AIDS immunopathogenesis, indicating hyper immune activation as a fundamental driving force of HIV-associated immune dysfunction.

Immune activation in the context of HIV infection is a broad term encompassing several features associated with cell activation, proliferation, differentiation and death, as well as the secretion of soluble molecules, and their subsequent effects [269]. HIV-infected individuals present elevated markers of immune activation, and associated disturbances, on $CD4^+$ and $CD8^+$ T cells [217, 270], as well as on B cells [271, 272], monocytes [273] and dendritic cells [274-276]. High levels of pro-inflammatory cytokines, such as TNF-α and IL-6, and chemokines, like macrophage inflammatory protein-1β (MIP-1β) and monocyte chemoattractant protein-1 (MCP-1), have also been described during HIV infection [277-280].

"Dr. Jeckyll, Mr. Hide": the pros and cons of heightened immune activation

Immune activation in response to HIV infection is essential to mount host immune responses, which are of utmost importance for viral suppression, both during the primary phase and the asymptomatic period of infection [126]. Paradoxically, this may also provide an immunological environment that actually drives viral replication [102]. Additionally, increased immune activation is associated with major lymphocyte disturbances, including increased activation-induced cell death (AICD), alterations in cell trafficking, and progressive disruption of microenvironments required for hematopoiesis and lymphocyte homeostasis [102, 135, 281-283].

Hence, persistent immune activation, not only derives from, but also compels HIV/AIDS disease progression.

2.1. Underlying mechanisms

The mechanisms underlying persistent immune activation during HIV infection are not fully clarified, but are likely to be multiple and include factors other than the direct impact of viral replication.

"Adding fuel to the fire": direct effects of the virus and associated host immune responses

HIV is able to directly activate cells of the innate immune system, particularly plasmacytoid dendritic cells (pDC), through direct binding to Toll-like receptors (TLR) [284, 285]. The upregulation of costimulatory and maturation marker expression on pDC, which constitute the first line of immune defense against viral infection, leads to 1) the production of type I interferons with both immune-stimulating and anti-viral activity [286]; 2) the bystander maturation of myeloid DC (mDC) [287] with the resulting enhancement of antigen-presenting functions, and 3) the overall stimulation

of T and B lymphocytes [288, 289], resulting in the activation of adaptive HIV-specific immune responses (both cellular and humoral).

During primary HIV-1 infection, robust specific T-cell responses are induced, in particular cytotoxic $CD8^+$ T cells, which play a key role in viremia decline and viral set point establishment [290]. These HIV-specific T-cell responses persist at high levels during chronic infection, being continuously boosted by the antigen (ag) produced during HIV replication [291, 292]. Nevertheless, HIV-specific $CD4^+$ T cells are present at a lower magnitude, possibly due to their preferential loss due to HIV infection [135]. Of note, in contrast to HIV-1 disease, HIV-2 infection seems to feature a better maintenance of HIV-specific $CD4^+$ T-cell responses, both in frequency and in quality [293-297], and an overall preservation of polyfunctional HIV-specific T-cell responses [298]. These features may contribute to the better HIV-2 disease prognosis [146].

The antibody response to HIV following infection is largely ineffectual, with early responses being mostly directed against non-neutralizing epitopes of the viral envelope [299, 300] and later responses being unable to keep up with the rapidly diversifying virus [301, 302]. Of note, several studies have suggested a better ability of HIV-2-, in comparison with HIV-1-infected individuals, to generate and preserve significant levels of circulating HIV neutralizing antibodies during the chronic phase of the disease [303-307]. These findings have been suggested to be related to particular conformations of the HIV-2 envelope proteins that may favour the triggering of potent neutralizing antibody responses [308].

Although virus-specific adaptive immune responses can be beneficial, contributing to the suppression of viral replication, they may also promote the inflammatory state associated with HIV infection [145].

Additionally, HIV proteins may play a direct role by inducing the activation of lymphocytes and monocytes/macrophages, as well as the production of pro-inflammatory cytokines and chemokines [269, 309], thereby promoting viral replication. In particular, gp120 may be able to activate immune cells, or enhance their responsiveness to activation, even in the absence of direct infection, through the

binding of CD4 or coreceptors, resulting in the induction of intracellular signaling pathways [310-314]. Another viral protein, Nef, is also able to induce lymphocyte activation, either directly [315-317] or through its action in infected macrophages [318-320].

"Several means justify one end": indirect effects of viral replication

Other factors besides the direct effects of HIV replication have been suggested to contribute to persistent immune activation, as listed below.

Unrelated infections: overall immune disturbances observed during HIV disease may result in suboptimal immune control of additional pathogens, including, but not limited to, those causing opportunistic infections during the later disease stages. Thus, reactivation of latent forms of cytomegalovirus (CMV) and Epstein-Barr virus (EBV) [321, 322], for example, ultimately lead to additional antigenic stimulation, and may further contribute to augment the level of immune system activation.

Microbial translocation: the massive HIV-associated depletion of $CD4^+$ T cells from the GALT [323] contributes to disrupt the integrity of the gut's mucosa, resulting in microbial translocation from the gut to the blood [324, 325]. HIV-1 infection has been associated with a significant increase in plasma lipopolysaccharide (LPS) levels, an indicator of microbial translocation that directly correlates with measures of immune activation [197]. Microbial translocation has been reported to primarily result in a profound activation of the innate immune responses [273, 326], as bacterial products such as LPS, flagellin and CpG constitute TLR ligands that directly stimulate peripheral monocytes/macrophages and DC to produce pro-inflammatory cytokines, whilst also interfering with adaptive immune responses [327]. Overall, sustained microbial translocation has been suggested to contribute to generalized activation and differentiation of lymphocytes and monocytes, being associated with: 1) markers of disease progression and severity in HIV-1 and HIV-2 infections [328]; and both 2) poor $CD4^+$ T-cell recovery [329, 330] and 3) residual viral replication [331] in the context of antiretroviral therapy. Recently, microbial translocation has also been suggested to

predict disease progression of untreated HIV-infected patients with high CD4+ T-cell counts [332]. However, although this process seems to play an important role in HIV pathogenesis in the developed world, it is unclear whether it contributes to HIV disease in African cohorts. Of note, while, in some African studies, no associations have been reported between circulating LPS and progression to AIDS [333, 334], in others, microbial translocation has been described as a major driving force of chronic inflammation in HIV disease [335, 336].

Heightened cytokine production: the increased production of pro-inflammatory cytokines, primarily induced at the level of innate immune responses to viral replication, may mediate non-antigen specific "bystander" activation of T and B lymphocytes. This "bystander" activation also involves the up-regulation of apoptosis-related molecules (such as the Fas molecule, CD95) on the surface of lymphocytes, rendering them more prone to AICD and significantly contributing to immune disturbances during HIV/AIDS progression [337-339]. Importantly, in addition to enhancing T-cell activation, some pro-inflammatory cytokines, such as IL-1β, IFN-α and TNF-α, may also decrease trans-epithelial resistance in mucosal tissues [340], thus promoting microbial translocation and contributing to further immune activation.

CD4+ regulatory T-cell (Treg) loss/dysfunction: an additional factor with the potential to affect hyper immune activation associated with chronic HIV disease is the reported depletion and/or functional impairment of CD4+ Treg cells observed in some studies [341, 342]. Although the results regarding the Treg compartment in HIV infection are conflicting [343, 344], as discussed in more detail later, an impairment of the known ability of Treg to suppress immune activation either through direct cell-to-cell contact, production of anti-inflammatory cytokines or inhibition of DC activity, would be very relevant in HIV/AIDS pathogenesis.

2.2. Consequences

The state of HIV-associated chronic immune activation and inflammation has wide-ranging deleterious effects on the immune system.

A direct consequence of T-cell activation is the enhancement of integrated virus transcription, with subsequent production of new virions with the ability to infect new target cells [345]. A vicious cycle is then established, in which HIV replication promotes immune activation and immune activation potentiates viral replication and the inherent consequences of viral mutation and evolution.

Chronic T-cell activation results in persistent turnover and promotes the differentiation of naïve into memory-effector cells. This contributes to the depletion of naïve T-cell subsets and skewed T-cell differentiation, leading to an over-accumulation of T cells at intermediate/end stages of differentiation during the course of HIV disease [346-349]. Importantly, although a large number of T cells end up dying upon activation, the dynamics of activation, expansion and apoptosis seem to differ for $CD4^+$ and $CD8^+$ lymphocytes [350-352]: while $CD8^+$ T cells experience extensive expansion upon activation and can establish a stable pool of resting memory cells, the capacity of $CD4^+$ T cells to expand and survive seems to be lower, such that the vast majority of activated $CD4^+$ T cells undergo apoptosis rapidly. Hence, the ultimate consequence of immune activation is a marked depletion of $CD4^+$ T cells, which constitutes the hallmark of HIV disease progression.

Polyclonal B-cell activation and hypergammaglobulinemia are also hallmarks of HIV infection [353]. HIV-1-related chronic immune activation has been shown to profoundly affect B cells, being associated with progressive impairments of specific humoral responses, loss of memory B cells, massive B-cell apoptosis and loss of germinal centres in lymphoid tissue [339, 353-359]. Of note, TGF-β has been reported to mediate collagen deposition and tissue fibrosis [360, 361], leading to structural effacement of lymph node architecture during HIV-1 and pathogenic SIV disease, thereby possibly contributing to B-cell disturbances, and impaired reconstitution of the B cell compartments upon ART [353, 362].

Importantly, the innate immune system is also significantly dysregulated by persistent immune activation [128, 285]. Disturbances regarding cell numbers, activation, function and survival of pDC, mDC and monocyte subsets have been extensively reported during the course of HIV disease [275, 363-371]. Defects in the

innate immune system are likely to be of major importance in the pathogenesis of HIV disease, not only by facilitating viral replication and contributing to defective adaptive responses, but also through the promotion of inflammation [285].

2.3. Counteractive responses

The immune system has developed specific regulatory mechanisms to prevent the deleterious effects of immune activation-driven pathology resulting from unchecked immune responses. One of these mechanisms is the up-regulation of inhibitory or immune checkpoint molecules, such as Programmed-Death 1 (PD-1) and T-cell Immunoglobulin and Mucin domain-containing molecule 3 (TIM-3), which dampen T-cell responses by different processes; another consists of the generation of cell populations with immunosuppressive capacity.

2.3.1. Inhibitory pathways – PD-1 and its ligands

Although having been initially described as a molecule preferentially expressed on dying cells [372], PD-1 (also known as CD279) was one of the first inhibitory receptors identified, with its important inhibitory function being revealed by the autoimmune-prone phenotype of *pdcd1*-/- mice [373, 374].

A member of the CD28 family [375], PD-1 is a 288 amino acid (aa) type I transmembrane protein composed of one immunoglobulin (Ig) super-family domain, a 20 aa stalk, a transmembrane domain, and an intracellular domain of 95 aa residues containing both an immunoreceptor tyrosine-based inhibitory motif (ITIM), and an immunoreceptor tyrosine-based switch motif (ITSM) [376, 377]. PD-1 is expressed on both lymphocytes and cells of the myeloid lineage [375, 376]. Of note, although not expressed on resting T cells, PD-1 may be induced upon activation, either by direct TCR engagement [376, 378, 379], or by the effect of commom γc cytokines, such as IL-2, IL-7, IL-15 and IL-21 [380].

There are two identified PD-1 ligands, PD-L1 (B7-H1; CD274) [381] and PD-L2 (B7-DC; CD273) [382], both consisting of type I transmembrane glycoproteins with short cytoplasmic tails containing no known motifs for signal transduction [376, 383]. PD-L1 is constitutively expressed on activated T cells and antigen-presenting cells, APC [384], as well as on B cells [385] and natural killer, NK, cells [386]; whereas PD-L2 is mainly restricted to DC and monocytes [384] and, more recently, also described on activated T cells [387, 388]. Both PD-1 ligands' expression has been shown to be induced by IFN-γ [384] and commom γc cytokines [380]. Importantly, as PD-1 and its ligands are expressed on the same cell types, bidirectional signaling may occur.

"Keeping cells on a diet": inhibitory signaling through the PD-1 receptor

Inhibitory receptor signaling typically attenuates immune responses, as widely described in T cells [376]. Whilst TCR engagement enables T cells to switch from quiescent into actively expanding cells, co-stimulation is thought to be required for the glucose uptake necessary for cell division and effector differentiation [377, 389]. Co-stimulation, by activating phosphatidylinositol-3-kinase (PI3K), and its downstream target Akt (protein kinase B), leads to increased expression of glucose transporters on the plasma membrane and general up-regulation of glycolytic enzyme activity [390]. PD-1 engagement leads to PI3K activation blockade, thereby impairing T-cell activation by denying the cell the resources required for cell division, effector function and differentiation [391] (Figure 11). Moreover, PD-1 ligation and subsequent interference with early activation events inhibits 1) the induction of the cell survival factor Bcl-xL [392]; 2) IL-2 production [393, 394]; 3) and the expression of transcription factors associated with effector cell function [376]. Of note, PD-1 ligation inhibits antigen receptor signaling only in *cis* and not in *trans* [395], indicating that PD-1 ligation must occur close to the site of antigen receptor engagement, an idea supported by the redistribution of PD-1 from uniform cell surface expression to the synapse during T-cell–APC interactions [396].

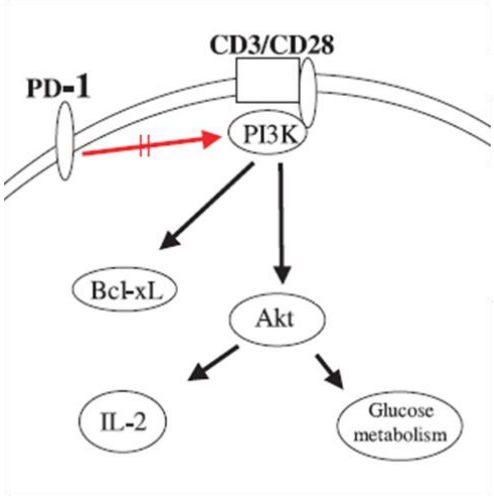

Figure 11. The inhibitory effects of PD-1 signaling in T cells. PD-1 engagement on the cell surface leads to phosphorylation of PD-1 cytoplasmic tyrosine residues, promoting the binding of phosphatases. This, in turn, interferes with phosphorylation of the PI3K pathway, affecting downstream signaling through Akt. PD-1 engagement ultimately decreases the induction of cytokines and cell survival molecules, also causing disturbances in glucose metabolism. Adapted from *Riley, Immunological Reviews 2009*.

PD-1 signaling typically has greater effect on cytokine production than on cellular proliferation, with significant effects on IFN-γ, TNF-α and IL-2 production [376]. Importantly, PD-1-mediated inhibitory signals depend on the strength of TCR stimulation, with greater inhibition being achieved at low levels of TCR signaling. This inhibition can be overcome by co-stimulation through CD28 [381] and/or IL-2 [393].

Of note, while many studies have shown that PD-Ls engagement can drive the inhibition of T-cell proliferation and cytokine production, others have found PD-Ls to enhance T-cell activation [397-400]. The reasons for these contradictory results are not yet clear and remain controversial, with some data suggesting that PD-L1 can increase T-cell proliferation by inhibiting IFN-γ-induced nitric oxide production by macrophages, a factor that can inhibit T-cell proliferation [401]. In addition, apart from influencing immune responses by binding to PD-1, PD-L1 has been shown to be able to interact specifically with B7-1 (CD80) to inhibit T-cell responses [402], further suggesting that bidirectional signaling may occur.

"Exhaustion vs. collateral damage control": the PD-1/PD-L1 pathway in HIV infection

PD-1 and its ligands play important roles in controlling immune responses against pathogens that cause acute and chronic infections – such as HIV –, being of

potential relevance for mediating the delicate balance between effective immune responses and immune-mediated tissue damage.

In the context of chronic HIV-1 infection, PD-1 up-regulation was first described as a marker of cellular exhaustion on HIV-1-specific $CD4^+$ and $CD8^+$ T cells [403-405].

Exhausted T cells are usually defined as no longer able to proliferate and exert effector functions, such as cytotoxicity and cytokine secretion in response to antigen stimulation [406]. Exhaustion has been suggested to be a progressive and hierarchical process, with proliferation and CTL function being lost first, followed by the loss of IL-2, TNF-α and IFN-γ production capacity [407].

Of note, HIV-1-specific T-cell "exhaustion", defined on the basis of PD-1 expression, has been shown to be partially reversible upon blockade of the PD-1/PD-L1 pathway [394, 403-405]. Moreover, not all exhausted T cells have been described as expressing PD-1 [404] and not all $PD\text{-}1^+$ cells have been shown to feature functional impairment [405, 408], suggesting an additional role of other inhibitory molecules for the true definition of T-cell exhaustion in the context of HIV infection (as will be discussed later).

Importantly, the frequency of $PD\text{-}1^+$ HIV-1-specific T cells has been extensively shown to directly correlate with markers of disease progression and severity [404, 409-411], decreasing in the context of ART-mediated immune activation reduction [412, 413] and viral load suppression [403, 404, 414]. This reinforces a possible major role of the PD-1/PD-L1 pathway during HIV pathogenesis.

A growing body of evidence now suggests that increased PD-1 levels may, instead of reflecting immune exhaustion, serve as a mechanism to control tissue damage [415, 416] driven by chronic immune activation [417] and/or persistent antigenemia associated with ongoing viral replication, even at low levels [292, 418]. In agreement with this view, PD-1 expression has been documented to depend on both T-cell activation and differentiation status, with central and effector memory populations, which include the majority of HIV-1-infected cells [419], and not the

terminally differentiated ones, expressing the highest levels of the inhibitory molecule [408, 420, 421],

"It takes (at least) two to tango": cooperation of PD-1 and TIM-3 during chronic HIV infection

TIM-3 expression has recently been reported to identify a highly dysfunctional, non-proliferating, no cytokine-producing, population of activated T cells, with highly elevated frequencies in progressive HIV-1 disease [422].

TIM-3 is an Ig super-family member that differs from other inhibitory molecules in that its activation-mediated up-regulation occurs specifically on T cells that have differentiated to produce IFN-γ ($CD4^+$ T helper 1 and $CD8^+$ T cytotoxic 1 cells). Hence, it typically limits Th1 responses [423, 424]. Of note, recent studies suggest that TIM-3 may also act as a human monocyte inhibitor, with its blockade being associated with increased pro-inflammatory cytokine production upon TLR stimulation [425, 426].

The mechanisms underlying TIM-3 regulation are still poorly understood, with evidence suggesting its induction to be dependent on Th1-cell activation [423, 424], and the transcription factor Tbet [427], whilst also being potentially involved in the differentiation of Th1 *vs.* Th17 cells [428]. Engagement of TIM-3 by its only identified ligand (galectin-9, Gal-9, a lectin highly expressed by leukocytes, fibroblasts and endothelial cells in inflamed tissues [429, 430]), typically results in the apoptosis of $TIM\text{-}3^+$ cells [423]. How this engagement process leads to the inability of T cells to enter cell cycle and secrete cytokines remains unknown, as well as the mechanisms underlying the persistence of the $TIM\text{-}3^+$ population [428, 431-434].

TIM-3 has been observed to be co-expressed with PD-1 in the context of both chronic viral infection, including HIV disease [435], and cancer [436]. This co-expression was restricted to a small percentage of T cells featuring the most exhausted phenotype, whilst T cells expressing PD1 alone still retained some function [422, 435-438]. Hence, co-blockade of the PD-1 and TIM-3 signaling pathways has

been shown to be more effective in restoring exhausted T-cell function than targeting either pathway alone [436, 439]. These findings further support the idea that PD-1 expression, by itself, may not always be associated with cellular exhaustion, being insufficient to fully characterize it in the absence of additional inhibitory molecules with potentially synergistic effects.

2.3.2. $CD4^+$ regulatory T cells

$CD4^+$ regulatory T cells (Tregs) may also act as modulators of immune activation.

Tregs actively suppress effector T cells and inhibit immune-mediated tissue damage, being typically characterized by the expression of the Treg-restricted transcription factor forkhead box protein 3 (FoxP3) [440], high level IL-2 receptor (IL-2R) α chain, CD25, expression, and suppressor functions [441]. Tregs are known to express the above described inhibitory molecules PD-1, PD-L1 and TIM-3 [442, 443].

$FoxP3^+$ Tregs may be divided into two subsets: naturally occurring (nTregs) and induced Tregs (iTregs), with the first being generated in the thymus [444, 445], and the latter developing in the periphery from $FoxP3^-$ naïve $CD4^+$ T cells through a process that, *in vitro*, has been described to depend on IL-2 and TGF-β [446]. Although initial Treg activation requires antigen-specific TCR stimulation, once activated they do not require further TCR ligation, thus being able to inhibit T-cell responses in an antigen-nonspecific manner [447, 448].

"Teaching cellular yoga lessons": Treg-mediated immune suppression

FoxP3 negatively regulates IL-2 transcription and up-regulates CD25 expression, rendering exogenous IL-2 essential for Treg survival [449, 450]. The constitutively high expression of CD25 allows efficient absorption of IL-2 by Tregs, and the concomitant depletion of IL-2 from the local milieu and thus any nearby conventional T cells. Some studies have suggested that IL-2 consumption by Tregs may result in cytokine

deprivation-induced apoptosis of effector T cells [451]; alternatively, Tregs may inhibit IL-2 mRNA production in $Foxp3^-$ effector T cells [452]. Another factor that has been described to be important for Treg function is the anti-inflammatory cytokine IL-10. IL-10-producing $FoxP3^+$ T cells were found to be abundant in the intestinal *lamina propria* and thought to play a critical role in mucosal tolerance. Additional mechanisms used by Tregs to influence immune responses include, but may not be restricted to [453]: 1) direct interactions with DC (with Tregs down-regulating co-stimulatory molecules such as CD80 and CD86, and thereby "educating" DC to become less efficient); and 2) lysis of effector T cells, APC, or NK cells, with some data indicating that Tregs may use granzyme A and perforin to kill target cells [454].

"The Godfather": PD-1/PD-L1 effects on Treg induction and function

Tregs and the PD-1/PD-L1 pathway are both critical in inhibiting immune activation, with elimination of either one leading to uncontrolled T-cell responses. Of note, the PD-1/PD-L1 pathway has been reported to be involved in the ability of Treg to suppress immune responses [455, 456], with resting Tregs possibly retaining PD-1 in intracellular compartments and translocating it to the cell surface upon TCR activation [457]. Furthermore, PD-L1 engagement has been shown to: enhance and sustain FoxP3 expression, and thus suppressive function of established iTregs [458]; increase the *de novo* generation of $FoxP3^+$ iTregs from naïve $CD4^+$ T cells; and convert Th1 cells into Treg [459]. Interestingly, in the context of chronic infection, Treg proliferation and function seem to be inhibited by PD-1 signaling [460].

"A double-edged sword": the role of Tregs in HIV infection

Several groups have analyzed frequencies and function of Tregs in HIV-1-infected patients. Nevertheless, the results are still somewhat controversial, with some authors supporting the idea that Tregs may contribute to HIV-1 pathogenesis by suppressing HIV-specific immune responses [344, 461-463], whilst others suggest that

these cells may have a protective role in HIV pathogenesis by limiting immune activation [342, 343, 464, 465].

In the context of HIV-2 infection, an expansion of Tregs has been reported, that was observed to be more directly linked to $CD4^+$ T-cell depletion than to circulating viral load, supporting an apparently better preservation of circulating naïve and memory Tregs as compared with other $CD4^+$ T-cell populations in HIV/AIDS [466].

2.3.3. Additional immunosuppressive mechanisms

Additional mechanisms able to inhibit immune activation have been described, involving the production of inhibitory factors, such as indoleamine 2,3-dyoxigenase (IDO) and IL-10.

IDO is an intracellular enzyme that catalyzes the degradation of the essential amino acid tryptophan (Trp), and is produced at high levels by pDC, exerting a powerful suppressive effect on T-cell proliferation and activity [467]. IDO has a tolerizing function in physiological settings, whilst during chronic pathologic disorders it has been suggested to play a role in impairing efficient immune responses, hence favoring the persistence of infection [274]. In addition, IDO activity seems to be closely linked with Treg function: Tregs induce IDO expression by pDC, and IDO-expressing pDC promote Treg differentiation from naïve $CD4^+$ T cells [468, 469]. Of note, as HIV is a potent activator of pDC, high levels of IDO expression are induced during acute HIV infection, which are then maintained during the chronic phase, concomitant with an impaired adaptive anti-HIV-specific response [470-472].

Production of the anti-inflammatory cytokine IL-10 by APC has been shown to lead to T-cell response impairment in the context of chronic viral infection, with blockade of the interaction between IL-10 and its receptor resulting in restoration of T-cell function and viral clearance [473, 474]. In HIV-1, IL-10 levels have been reported to be elevated and associated with markers of disease progression [475]. Of note, it has recently been proposed that high amounts of microbial products (likely associated

with microbial translocation) and inflammatory cytokines in the plasma of HIV-1 infected individuals may direct PD-1 up-regulation by monocytes, leading to IL-10 production and reversible $CD4^+$ T-cell dysfunction [476].

"Trying to keep chaos under control": immunosuppressive effects of viral proteins

Paradoxically, some viral proteins have also been described as helping to contribute to the attenuation of T-cell responses, particularly in the context of HIV-2 infection. This may translate into an adaptive advantage for the virus, as it could allow a longer period of virus production to occur, by increasing the life span of infected cells, through immune activation reduction and apoptosis suppression.

HIV-2 Nef has been shown to down-modulate TCR-CD3 in infected T cells, thereby limiting their responsiveness to activation mediated by this complex [315]. Of note, this capacity has been proposed to have been lost during viral evolution in the SIV lineage that gave rise to HIV-1, likely resulting in its increased pathogenicity in humans [315]. Nevertheless, HIV-1 Nef has been shown to induce PD-1 expression during *in vitro* infection [477], demonstrating an important role of Nef in the regulation of inhibitory molecules.

Finally, viral envelope glycoproteins have been described to have immune-suppressive properties [478-482], with the HIV-2 envelope glycoprotein gp105 exerting stronger negative effects on T-cell activation than its HIV-1 counterpart, gp120 [483].

Part 3. Ability to replenish $CD4^+$ T cells in HIV infection

The immune system has the ability to reconstitute itself following profound depletion, through both thymic-dependent and -independent mechanisms. During HIV infection, there is a need to reconstitute an extremely depleted memory $CD4^+$ T-cell pool. This process depends, not only on the homeostatic proliferation of memory $CD4^+$ T lymphocytes, but also on the reconstitution of the memory $CD4^+$ T-cell pool with cells differentiating from the naïve compartment. Importantly, HIV-induced immunopathology directly limits the above renewal processes by impairing thymic function and disrupting lymph node architecture [484].

Notably, $CD4^+$ T-cell regenerative pathways seem to be significantly different from those underlying $CD8^+$ T-cell restoration. Indeed, whereas reconstitution of $CD8^+$ T-cell numbers, after T-cell depletion, has been shown to be rapid and mainly rely on peripheral expansion, recovery of $CD4^+$ T cells appears to be both more limited and slower, being critically dependent on the naïve $CD4^+$ T-cell pool and, therefore, constrained by the age-related decline in thymopoiesis [485, 486]. Furthermore, while a significant proportion of activated expanded $CD8^+$ T cells may later rejoin the resting memory $CD8^+$ T-cell pool, the fate of activated $CD4^+$ T cells seems to be different, with fewer being able to survive [352, 487, 488]. Finally, maintenance and homeostatic expansion of naïve $CD4^+$, but not $CD8^+$, T cells seems to be critically dependent on the presence of a preserved microenvironment of the secondary lymphoid tissue [489].

3.1. *De novo* $CD4^+$ T-cell production

The thymus is an encapsulated organ, comprised of thymic stroma and hematopoietic cells, and consisting of several lobules with distinct cortical and medullary areas [490]. Cortical and medullary thymic epithelial cells (cTEC and mTEC) constitute the main components of the thymic microenvironment, with each subset playing a distinct role in thymocyte development [490].

T cells are produced in the thymus from pluripotent $CD34^+$ hematopoietic stem cells (HSC), derived from the foetal liver (initially) and bone marrow [491, 492]. Double-negative (DN) $CD4^-CD8^-$ T-cell precursors give rise to double-positive (DP) $CD4^+CD8^+$ thymocytes, which then undergo selection, lineage commitment, and differentiation into naïve $CD4^+$ and $CD8^+$ single-positive (SP) T cells [493] (Figure 12). These latter steps are primarily determined by TCR signaling, and are dependent upon the interaction of CD4 and CD8 with major histocompatibility complex (MHC) class II and I molecules, respectively, expressed on thymic stromal cells [494].

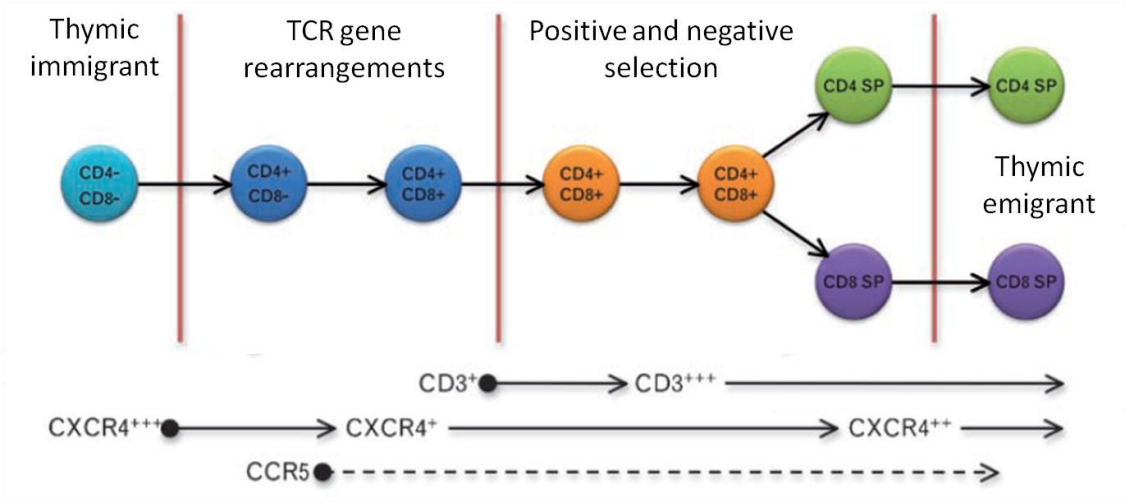

Figure 12. Simplified model of T-cell development. After entering the thymus, $CD4^-CD8^-CD34^+$ hematopoietic stem cells (thymic immigrants) undergo TCR gene rearrangements, by the end of which they acquire a fully functional TCR-CD3 complex. During this process, CD4 and CD8 molecules are also sequentially and progressively up-regulated, first generating an intermediate population comprising immature $CD4^+CD8^-$ single-positive cells; and subsequently a $CD4^+CD8^+$ double-positive thymocyte subset. $CD3^+CD4^+CD8^+$ thymocytes then go through a process of positive and negative selection, giving rise to mature $CD4^+$ and $CD8^+$ single-positive T cells that finally exit the thymus. CXCR4 and CCR5 molecule expression is also modulated during thymopoiesis, as indicated, with CXCR4 being found throughout all thymocyte subsets, and CCR5 being mainly restricted, although at low levels, to mature thymic emigrants. Adapted from *Fang et al, AIDS 2008*.

IL-7 has been identified as essential for human T-cell development, being highly produced by thymic epithelial cells [495-498]. The IL-7 receptor (IL-7R) is a heterodimer consisting of the IL-7Rα chain (CD127) and the common γ chain (γc, CD132), the latter subunit being expressed at all stages of thymocyte maturation, at varying levels [499]. Importantly, studies with exogenous IL-7 have shown it to be able to promote survival, proliferation and differentiation of developing thymocytes, depending on their maturation state [499-501].

Of note, although thymic function has been shown to decline with age, a substantial output of naïve $CD4^+$ and $CD8^+$ T cells appears to be maintained into late adulthood [502, 503], providing a T-cell pool with a broad TCR repertoire, potentially more able to respond to neo-antigens [504, 505].

"Burning down the nursery": thymic infection by HIV

Surface expression of the HIV coreceptors CXCR4 and CCR5 is also modulated during thymocyte development. Whilst CXCR4 is expressed throughout all thymocyte developmental stages (peaking at the immature $CD3^-CD4^+CD8^-$ single positive phase and being slightly up-regulated upon leaving the thymus – Figure 12), CCR5 expression is mostly restricted to a small proportion of thymocytes, being mainly observed on the surface of mature $CD4^+$ and $CD8^+$ single positive cells (and, in contrast to CXCR4, undergoing a mild down-regulation just prior exiting the thymus) [506]. Thus, both CCR5- and CXCR4-using HIV-1 strains have been reported to infect the thymus, being able to enter and complete reverse transcription in most thymocyte subsets [507]. Due to CXCR4's broad expression pattern, CXCR4-using viruses are able to establish productive infection in immature, as well as mature thymocytes, contributing to a rapid depletion of $CD4^+$ cells located within the cortex (more immature thymocytes) and medulla (mostly SP thymocytes) of the infected thymus [506]. Of note, as a consequence of the infection of immature thymocytes and subsequent thymocyte differentiation, HIV-1-infected $CD8^+$ single positive T cells are also found in the thymic tissue [508, 509], and in the peripheral $CD8^+$ T-cell population [510].

Importantly, thymocyte infection by HIV-1 has been recently reported to alter IL-7 responsiveness of thymocytes, independently of CD127 expression, thereby further contributing to the disruption of thymopoiesis [511] and the associated thymic involution [512-514] observed during HIV disease.

Of note, altered thymopoiesis during HIV-1 infection may be related, not only to thymic infection by the virus, but also to a compromised ingress of competent hematopoietic precursors into the thymus [515]. In fact, HIV-1 has been shown to

directly or indirectly affect HSC maturation in the bone marrow and, therefore, the production of several hematopoietic lineages [516-518]. In agreement, HIV-1-infected individuals often present abnormal bone marrow architecture and cellularity [519].

"After the damage is done…": thymic output assessment

Changes in thymic function may be evaluated by several methods [281], including thymus size and metabolic labeling assays and, more recently, quantification of T-cell receptor excision circles (TREC).

TREC are episomal DNA circles generated during intrathymic rearrangement of T-cell receptor α, β and δ *loci* that are not replicated upon cell division, existing at high levels in recent thymic emigrants (RTE) [502, 520]. Different types of TREC can be quantified in peripheral blood, namely: 1) DβJβ (β) TREC, which are created early in thymocyte maturation, during the first step of TCRβ *locus* organization; and 2) signal-joint (sj) TREC, that constitute the major byproducts of the TCRδ *locus* excision, and are generated at a later stage, after extensive intrathymic proliferation [502, 520].

The interpretation of data utilizing only sjTREC quantification has been proven to be difficult, with reduced sjTREC levels in the periphery having been suggested to simply reflect their dilution resulting from increased proliferation of the naïve T-cell pool [521]. Hence, the levels of sjTREC alone may not accurately mirror thymic output, since they may reflect, in part, cellular peripheral proliferative history. Conversely, the sj/β TREC ratio, which becomes fixed once sjTREC are generated, is not influenced by peripheral proliferation, and thus constitutes an indirect estimation of intrathymic proliferation and consequently a primary determinant of thymic output [520].

Of note, whilst the sj/β TREC ratio in peripheral T cells in HIV-1 infection has been shown to be significantly reduced [520], its maintenance in HIV-2 infected patients has been reported, suggesting a preservation of efficient thymopoiesis, even in older individuals [522]. This, in turn, may contribute to the better preservation of peripheral CD4$^+$ T cells and the associated slower disease course.

Several attempts have been made to phenotypically identify RTE within peripheral T cells. Thus, the use of specific surface markers has been suggested in order to differentiate the $TREC^{high}$ RTE-containing naïve T-cell populations from those consisting of $TREC^{low}$ cells that have undergone peripheral expansion. The first such marker to be identified was CD31 (platelet endothelial cell adhesion molecule-1, or PECAM-1) [523]. Peripheral naïve $CD4^+$ T cells expressing this molecule are highly enriched in TREC, containing the true RTE population. On the other hand, $CD31^-$ naïve $CD4^+$ T cells feature low to undetectable TREC levels [523]. More recently, protein tyrosine kinase 7 (PTK7) has been described as a marker of RTE [524], with $PTK7^+$ naïve $CD4^+$ T cells being uniformly $CD31^+$, containing higher levels of sjTREC, in comparison with their $PTK7^-$ counterparts (both $CD31^+$ and $CD31^-$), and rapidly decreasing after complete thymectomy.

Importantly, whilst memory $CD4^+$ T cells represent the primary target for infection by HIV in peripheral blood, naïve $CD4^+$ T cells also appear to significantly contribute to the infected T-cell population, with both RTE and non-RTE naïve $CD4^+$ T cells (defined based on the levels of CD31 expression) having been recently reported to be infected by HIV-1 *in vivo* [525]. Of note, no correlations were observed between the levels of RTE infection and intra-thymic precursor T-cell proliferation, suggesting that HIV-1 infection of immature thymocytes at early developmental stages makes a limited contribution to the circulating pool of infected naïve $CD4^+$ T cells [525].

"Under the influence": the impact of thymic function impairment on immune reconstitution during ART

Thymopoiesis is likely to contribute to $CD4^+$ T-cell reconstitution upon ART in HIV-1 infected patients [526-529]. In agreement, the baseline level of thymic function at the time of ART initiation has been positively correlated with evidence for thymic-dependent reconstitution capacity following ART [530, 531].

Of note, an important role for decreased thymic function in immune reconstitution has been suggested in HIV-1+ ART-discordant individuals [529, 532], an observation supported by evidence indicating that the incidence of immunologic discordance increases with age [529, 533]. As previously mentioned, whilst most HIV-1+ individuals achieve measurable thymic-dependent T-cell replenishment in response to ART [526-528], around 20% feature only limited $CD4^+$ T-cell recovery despite being able to achieve complete viral suppression [534].

3.2. Homeostasis of the peripheral $CD4^+$ T-cell pool – the fundamental role of IL-7

Following thymic export, RTE are incorporated into the peripheral naïve T-cell pool, thereby contributing to its preservation and maintenance of a diverse T-cell repertoire [535-537]. The mechanisms underlying this incorporation remain controversial [537-540], with some authors suggesting RTE to be preferentially incorporated only in the context of a depleted T-cell compartment [541]. Of note, subsequent cycling of RTE, ultimately leading to efficient post-thymic T-cell differentiation, has been shown to be highly correlated with IL-7 responsiveness [542-544].

"A helping hand": the IL-7/IL-7R axis in peripheral T-cell homeostasis

Besides playing an important role during thymopoiesis, IL-7 has also been described as a critical modulator of survival and homeostatic proliferation of both naïve and memory T cells [545].

In the periphery, IL-7 is mainly produced in the T-cell areas of secondary lymphoid organs (SLO) by fibroblastic reticular cells (FRC) [546]. IL-7 is constitutively produced, with T-cell responses to IL-7 being tightly regulated through the modulation of the IL-7Rα chain (CD127) [545].

CD127 is expressed on naïve T cells (Figure 13) [547] and its expression is down-regulated upon strong TCR activation, being lost on effector memory T cells and senescent memory populations, but re-expressed on the resting memory T-cell pool [547]. Furthermore, T cells have been shown to down-regulate CD127 after contact with IL-7 and, reciprocally, to up-regulate CD127 in the absence of the cytokine, a process involving new protein synthesis [548]. Such feedback control of CD127 expression directly contributes to the size of the peripheral T-cell pool: whilst most resting cells express CD127, which allows them to receive basal IL-7 signals for survival and proliferation, activation or IL-7 signaling leads to CD127 down-regulation, thereby preventing excessive expansion. Furthermore, IL-7-mediated CD127 down-regulation on mature T cells may serve to maximize the number of T cells that can respond to the limiting amounts of IL-7 available when normal T-cell numbers are present.

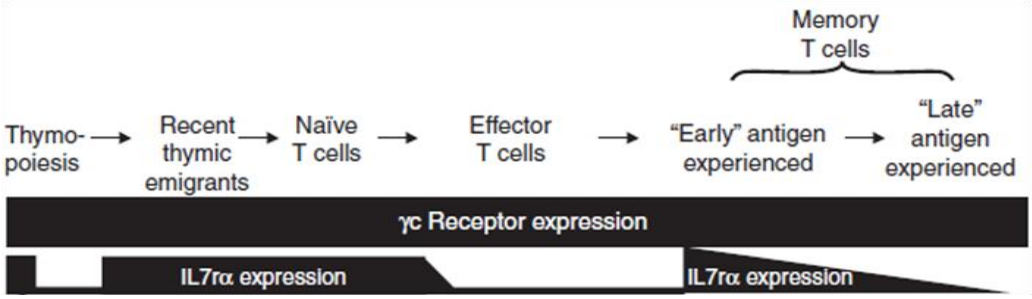

Figure 13. IL-7 receptor subunit expression during T-cell development. CD132 (γc receptor) expression is relatively constant, with the regulation of CD127 (IL-7Rα) expression largely dictating the availability of the IL-7 receptor. Adapted from *Capitini et al, Journal of Internal Medicine 2009*.

IL-7 signaling mediates T-cell survival and maintenance in several ways: *via* up-regulation of anti-apoptotic factors, such as Bcl-2 and Mcl-1 [549], which mediate the inhibition of pro-apoptotic molecules, such as Bax or Bim [550, 551]; maintaining longer telomeres by inducing telomerase activity [552]; and promoting cell proliferation, growth and metabolic activity by direct interaction with the PI3K pathway [553, 554].

"Getting a life after the thymus": naïve CD4+ T cells and IL-7

Once RTE are integrated into the naïve peripheral pool of a T-cell-replete host, they usually enter a resting state with no, or very slow, proliferation, being able to remain at interphase for many weeks or months [555]. Naïve cells require low-affinity self-peptide:MHC signals, as well as IL-7, for survival and persistence *in vivo*, signals most likely available within the lymphoid tissue [496, 556]. Importantly, increasing evidence suggest that the maintenance of naïve T-cell quiescence is actively maintained through the interplay of various negative regulatory mechanisms aimed at preventing cell activation and cycling [557]. The repression of NF-κB seems to be particularly important in this context, involving continuous synthesis of the NF-κB inhibitor IκB, mediated by members of the forkhead transcription factor family, whose expression is high in naïve T cells [558, 559]. Recently, Foxp1 has been shown to be critical for maintaining naïve T-cell quiescence, *via* the negative regulation of CD127 expression and signaling [560]. Furthermore, Tsc1-mediated suppression of mTOR, a central regulator of glycolysis and cellular metabolism, has also been shown to be crucial in actively sustaining the quiescence of naïve T cells [561]. Of note, unique patterns of chromatin condensation may also play a part in the preservation of the quiescent state, limiting the access of factors that are essential for peripheral T-cell proliferation to DNA, a feature that is only overcome upon TCR activation [562].

Despite the pronounced reduction in thymic function observed during ageing, a significant proportion of naïve CD4+ T cells are maintained in the elderly [563, 564]. This has been suggested to be related with homeostatic peripheral proliferation [538, 565], a process whereby naïve CD4+ T cells expand post-thymically, whilst retaining their naïve phenotype and functional characteristics. Hence, the appropriate homeostatic cues are likely to induce an attenuation of the negative regulatory pathways that maintain naïve T cells in a non-proliferative state, without resulting in "full" activation and consequent high level proliferation/differentiation.

This scenario implies the existence of, at least, two distinct subsets of human naïve peripheral CD4+ T cells differing in their proliferative history [565]: one subset highly enriched in RTE, and a second subset comprising naïve CD4+ T cells that have

undergone low-affinity self-peptide:MHC-dependent peripheral proliferation. As the high TREC level content of $CD31^+$ naïve $CD4^+$ T cells has been observed to be only slightly reduced in comparison with that of thymocytes, this subset is thought to be the one containing RTE [523]. On the other hand, the significantly reduced TREC content observed in $CD31^-$ naïve $CD4^+$ T cells, strongly suggests their more extensive peripheral proliferation [565]. In agreement, in healthy adults, $CD31^+$ naïve $CD4^+$ T cells have been shown to display a polyclonal TCR repertoire, whereas $CD31^-$ naïve $CD4^+$ T cells are characterized by a more restricted TCR repertoire [566]. Moreover, the progressive age-associated decline in naïve $CD4^+$ T cells has been reported to be mainly due to a reduction in the $CD31^+$ naïve subset, whilst the $CD31^-$ subset persists, further supporting the involvement of thymic output in the maintenance of $CD31^+$ cells [566, 567].

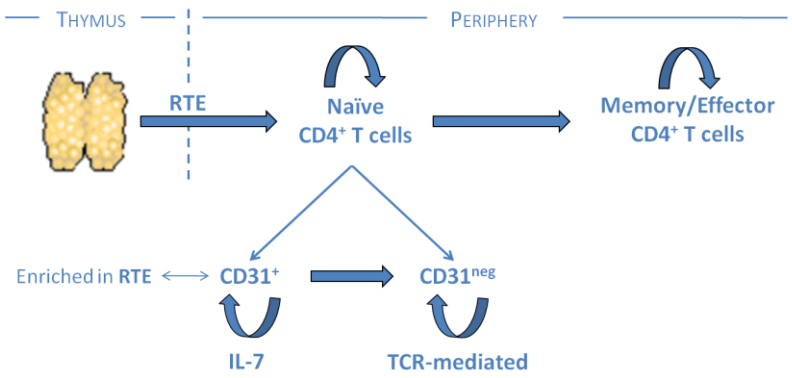

Figure 14. Post-thymic proliferation of human naïve $CD4^+$ T cells. After leaving the thymus, RTE are incorporated into the pool of $TREC^{high}CD31^+$ naïve $CD4^+$ T cells that later originate the $TREC^{low}CD31^-$ naïve $CD4^+$ T-cell subset. Upon cognate interaction with foreign antigens, both naïve subsets can then differentiate into memory/effector $CD4^+$ T cells. Whilst IL-7 has a major role in maintaining the $CD31^+$ naïve $CD4^+$ T-cell pool, $CD31^-$ naïve $CD4^+$ T-cell preservation mainly relies on low-affinity self-peptide:MHC-TCR signaling.

Importantly, IL-7 has been recently described to preferentially expand the $CD31^+$ subset of adult naïve $CD4^+$ T cells, without loss of CD31 expression, in a TCR-independent way, whilst promoting the preservation of CD31 on non-proliferating cells [568]. This indicates that, in adulthood, IL-7 may play a preferential role in the maintenance of $CD31^+$ naïve $CD4^+$ T cells, a population that is known to include RTE and, in this way, contribute to the preservation of TCR repertoire diversity within the

naïve CD4+ T-cell pool (Figure 14). Furthermore, apart from having been shown to be sufficient to induce widespread cycling of naïve T-cell populations, IL-7 also appears to contribute to the trafficking of RTE to lymph nodes [569], where they have optimal access to IL-7. Besides inducing TCR-independent homeostatic proliferation of CD31+ naïve CD4+ T cells [568], IL-7 may also favour low-affinity self-peptide:MHC-mediated cell cycling [545].

"Life goes on": memory CD4+ T cells and IL-7

Activation by foreign antigens induces CD4+ naïve T cells to undergo massive expansion for several days, with the concomitant acquisition of the appropriate effector functions [570]. Most effector cells die shortly afterwards, but a small fraction of them survive almost indefinitely as memory CD4+ T cells [571].

Although regulation of the overall pool size, under T-cell-replete conditions, applies to both naïve and memory CD4+ T cells, distinct mechanisms appear to control homeostasis of the two cell types. Hence, in contrast to what has been suggested for naïve CD4+ T cells, maintenance of memory CD4+ T cells seems largely independent of MHC-mediated signaling [496], and it appears to mainly rely on a combination of IL-7 and IL-15, both for survival and intermittent homeostatic proliferation [572-574]. Foreign antigens have been suggested to play an indirect role, for example, through activation of the innate immune system, thereby leading to production of the key homeostatic cytokines required by memory CD4+ T cells [571].

"The circle of life": apoptotic cell death and IL-7

As T-cell homeostasis implies the maintenance of a constant number of cells, it is essential that thymic output and peripheral T-cell expansion are balanced by lymphocyte death. Hence, under such conditions where cytokines promote the proliferation of specific T-cell subsets, others must die.

In this respect, IL-7 has been shown to induce Fas (CD95) expression on naïve $CD4^+$ T cells, rendering them highly susceptible to Fas-induced cell death, a process enhanced by the presence of IL-2 [575]. Of note, this susceptibility to apoptosis was not correlated with proliferation *per se* but, rather, with the maturation state of the cell, with RTE dying at the highest levels [575]. As RTE are, as previously mentioned, included in the $CD4^+$ naïve T-cell subset that is preferentially expanded by IL-7, increased susceptibility to Fas-induced cell death appears to constitute an important mechanism for the control of IL-7-mediated naïve $CD4^+$ T-cell proliferation [576].

"Trying to avert a major crisis": IL-7 signaling-mediated homeostasis in the context of HIV-related lymphopenia

Under normal circumstances, physiological use of IL-7 leads to stable levels of the cytokine. However, in lymphopenic states such as chronic HIV infection, circulating IL-7 levels rise, mainly due to a diminished consumption associated with reduced cell targets [577]. Accordingly, circulating IL-7 levels have been reported to be increased during HIV-associated lymphopenia, in direct association with $CD4^+$ T-cell depletion [578, 579] and reduced CD127 expression on T cells [580-582]. Importantly, in comparison with HIV-1-infected counterparts, HIV-2-infected patients have been shown to feature a delayed increase in IL-7 levels during disease progression, as well as a better preservation of CD127 expression, particularly in the naïve $CD4^+$ T-cell pool, suggesting a maximization of available resources in the context of this disease [583].

When in excess, IL-7 may be able to induce extensive naïve $CD4^+$ T-cell expansion to self-antigens by amplifying the weak TCR signaling resulting from contact with self-peptide:MHC ligands, a feature that may promote autoimmunity [565, 584]. Additionally, IL-7 has been shown to induce naïve $CD4^+$ T-cell TCR signaling-independent proliferation [568]. Moreover, a component of the homeostatic response may also rely on induction by foreign antigens, particularly in the context of chronic HIV infection. Hence, the resulting overall enhancement in cellular proliferation may

also render T-cell-depleted patients at a greater risk of lymphoproliferative disorders or lymphoma [585].

"Pulling the plug": disruption of secondary lymphoid tissues during HIV infection

As ongoing viral replication occurs and chronic immune activation increases, pathological changes are induced in lymph node architecture in HIV-1-infected individuals [135]. Indeed, as disease progresses, lymph node structure effacement occurs and $CD4^+$ T-cell population becomes depleted [586, 587]. Importantly, preservation of this niche is essential to ensure proper function of the homeostatic mechanisms maintaining the naïve $CD4^+$ T-cell pool, as well as the productive interactions between T cells and APC that are involved in generating and maintaining memory $CD4^+$ T cells. Thus, over time, damage by the virus to the supply routes and anatomical niches that maintain the resting $CD4^+$ T-cell compartments act together with the homeostatic strains imposed by chronic immune activation to further exacerbate the progressive loss in $CD4^+$ T-cell numbers.

Recently, a model has been proposed in which collagen deposition and loss of the FRC network impede access to, and destroy the source of, IL-7 during HIV-1 infection [283, 361]. As previously mentioned, naïve T cells depend on IL-7, produced by the FRC network, for survival. As HIV-1 infection progresses, chronic immune activation elicits a TGF-β-mediated Treg counter-response, and consequently, increased collagen production and deposition [360]. This sets in motion cooperative and cumulative mechanisms that contribute to progressive T-cell depletion [283, 361]: collagen deposition disrupts T-cell access to IL-7 provided on the FRC network, leading to increased apoptosis of T cells, particularly naïve $CD4^+$ T cells; whilst, in turn, the decreased availability of T-cell-derived lymphotoxin-β (LT-β), on which FRC cells depend, further contributes to FRC network impairment. As a consequence, naïve $CD4^+$ T cells are diminished in proportion to lymphoid tissue structure destruction, from early stages of HIV-1 infection. Because of the progressive and cumulative nature

of this pathological process, apoptosis of naïve $CD4^+$ T cells continues at elevated levels after ART initiation, with damage to lymphoid tissue architecture being predictive of the degree of immune reconstitution achieved upon antiretroviral treatment [283, 588].

3.3. Immune senescence

A general deterioration of the immune system occurs over time, possibly as a consequence of lifelong exposure to pathogens, leading to immune senescence. This may be, in part, responsible for the increased incidence and/or rapid progression of many infectious diseases that are observed during ageing [589].

One of the characteristic features of immune senescence is the erosion of the T-cell compartment due to the combination of age-associated thymic involution and replicative senescence, a phenomenon describing the irreversible state of growth arrest that occurs following multiple rounds of cell division [590]. This so-called "Hayflick limit" [591] has been described extensively for both $CD4^+$ and $CD8^+$ T lymphocytes [592], with memory T cells presenting a significantly reduced replicative potential as compared to naïve T cells from the same individual [593, 594].

"Holding back the years": telomeres and telomerase

Telomere length constitutes a commonly used marker of replicative history. Localized at the end of linear chromosomes, telomeres consist of short, tandem 5'-TTAGGG-3' repeats, associated with specific proteins [595]. Telomere functions include the stabilization and protection of chromosomal ends from events such as degradation, illegitimate recombination and fusion with other telomeres or DNA ends; determination of chromosomal localization within the nucleus; and overall regulation of cellular replicative capacity [596]. In the absence of telomerase (see below), telomeres shorten by, approximately, 100 base-pairs *per* cell population doubling,

resulting in chromosome instability and, eventually, growth arrest and/or apoptosis [597]. A size of 5-7 kb is considered indicative of replicative senescence [597]. Hence, telomere length, not only provides a tool for the analysis of the replicative history, but is also predictive of the remaining proliferative potential of the cells.

Telomerase is a ribonucleoprotein enzyme that synthesizes telomeric repeats using its own telomere sequence-specific RNA template, and thus helps prevent excessive telomere erosion during successive cell divisions [596]. Telomerase consists of two core components: a catalytic subunit (telomerase reverse transcriptase, TERT) and a telomerase RNA component (TERC) [598]. Although TERC is ubiquitously expressed, the expression of TERT is tightly regulated and is considered a limiting factor for telomerase function [599]. Most normal somatic cells do not express telomerase, which is only active in germ cells, during embryogenesis, in adult stem cells and in activated immune cells [600]. In T cells, although little or no telomerase activity is detected in the resting state, high levels are induced upon stimulation *via* TCR cross-linking or cytokines, including IL-7 [552, 601-603]. Of note, while telomerase activity may compensate for telomere loss, its induction in activated T cells is only transient [604], and lost upon repeated stimulation [605, 606]. This ultimately leads to telomere erosion and replicative senescence, with the concomitant and progressive accumulation of terminally differentiated T cells with short telomeres.

Importantly, although loss of cell proliferation is a characteristic feature of replicative senescence, T cells in this state are still able to maintain some normal functions [592]. Hence, while senescent CD8$^+$ T lymphocytes have been shown to retain normal antigen-specific cytotoxicity, senescent CD4$^+$ T cells are able to secrete some cytokines after TCR stimulation, in an antigen-specific fashion [607]. Furthermore, evidence has also arisen for the acquisition of additional functions, including MHC-unrestricted cytotoxicity (natural killing) and suppressive effects on other T cells [608]. Thus, replicative senescence does not constitute a general breakdown of normal function but, rather, may comprise selected genetic and phenotypic alterations, resulting not only in loss, but also in gain of some functions [592].

"Getting prematurely old": replicative senescence and HIV/AIDS

Several immunological alterations that characterize HIV infection, which is associated with increased cell proliferation, are remarkably similar to those naturally accumulated over time in HIV-uninfected elderly individuals [609, 610]. In agreement, telomere length of total peripheral blood mononuclear cells (PBMC) from HIV-1-infected individuals has been observed to shorten at an increased rate in comparison with age-matched seronegative controls [611]. Furthermore, this accelerated telomere reduction was found to correlate with HIV/AIDS disease progression, supporting an association between an increased ageing of the peripheral immune system and T-cell loss, that ultimately leads to AIDS [611].

Importantly, although telomere shortening during HIV-1 disease has been consistently observed within the $CD8^+$ T-cell subset [612-614], data on $CD4^+$ T cells remain controversial, with increased, decreased and stable telomere length having been reported for this particular subset [615-617]. These findings support different telomere length dynamics in the two T-cell subsets [618], with $CD4^+$ T-cell telomere shortening possibly occurring only at later disease stages [615, 619].

There are several possible explanations for the differences observed in telomere length between $CD4^+$ and $CD8^+$ T cells during the course of HIV-1 disease [620]. HIV-infected $CD4^+$ T cells may undergo only few divisions before dying, thereby limiting the time frame during which telomere shortening can occur. Moreover, when activated $CD4^+$ T cells are replaced by thymus-derived naïve cells, the longer telomeres of the naïve population may obscure any shortening that might have occurred in the memory $CD4^+$ T-cell subset. Another possible explanation concerns the potential involvement of telomerase in the different telomere length dynamics of $CD4^+$ and $CD8^+$ T cells. *In vitro* experiments have failed to show any differences in baseline or inducible telomerase activity between $CD4^+$ and $CD8^+$ T cells in HIV-infected individuals [614, 617, 621]. These data, obtained from HIV-infected

individuals in the chronic phase of the disease, do not preclude, however, a possible *in vivo* involvement of telomerase at an earlier point in the disease process.

The advances in the understanding of HIV/AIDS immunopathogenesis made over the last 30 years have been extremely important. Nevertheless, further elucidation of the mechanisms underlying chronic immune activation and impairment of $CD4^+$ T-cell replenishment during HIV infection is essential to achieve a "functional cure" that will allow ART discontinuation.

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CHAPTER 2:
AIM AND WORK PLAN

This work investigated the responses mounted by the immune system in order to cope with the challenges imposed by chronic HIV disease, using HIV-2 infection as a strategy to study HIV/AIDS-related immunopathogenesis. As a naturally-occurring form of attenuated HIV disease, HIV-2 infection provides an important model that can facilitate the identification of factors related to a more efficient immune response, and better disease prognosis, potentially providing a rationale for novel therapeutic interventions.

Although a hyper-activated immune system has long been recognized as a hallmark of HIV infection, closely linked to the continuous $CD4^+$ T-cell depletion that characterizes HIV/AIDS, the mechanisms underlying progressive immune activation remain poorly understood.

Of note, in spite of the approximate two-log difference in plasma viral load, HIV-1- and HIV-2-infected individuals with the same degree of $CD4^+$ T-cell depletion feature comparable levels of immune activation. Nevertheless, rates of disease progression are clearly distinct, with HIV-2 infection having a limited impact on the mortality of infected adults.

Innate immunity plays an important role in acute HIV infection, helping limit viral replication, whilst promoting the development of adaptive immune responses. During the course of chronic HIV disease, innate immune cells may act as modulators of overall immune activation. Thus, we hypothesized that the distinct course of HIV-1 and HIV-2 infections may be related to the quality of the induced innate immune responses. Furthermore, it is likely that differences in the ability to replenish the $CD4^+$ T-cell compartment also contribute to the distinct rate of disease progression that is observed in the two infections.

Therefore, this work aimed to address the following specific questions in HIV/AIDS pathogenesis:

Specific Aim 1. Is the relatively "benign" course of HIV-2 infection associated with distinct monocyte and mDC imbalances? Do these relate to a putative increase in the translocation of microbial products, such as LPS, as described for HIV-1 infection?

These results are described in Chapter 3.1.

Specific Aim 2. Progressive $CD4^+$ T-cell depletion is linked to immune activation in both HIV-2 and HIV-1 infections. Nevertheless, the rate of decline is much slower in HIV-2 disease. Is this associated with an up-regulation of inhibitory molecules that may limit HIV-associated immune activation and immunopathology?

Results regarding the PD-1/PD-L1 pathway in monocytes and mDC are described in Chapter 3.1., whereas those related to T cells are in Chapter 3.2.

Specific Aim 3. Replenishment of the $CD4^+$ T-cell pool is critically dependent on the homeostasis of the naïve compartment. We addressed two main questions in this context:

a) Does the homeostatic response of naïve $CD4^+$ T cells during prolonged HIV-2 disease impact on telomere length? (Chapter 3.3.)

b) Is the response of naïve $CD4^+$ T cells to IL-7 stimulation preserved in HIV-2-infected individuals? (Chapter 3.4.)

Specific Aim 4. HIV-1-associated immunodeficiency also features significant disturbances of the B-cell compartment. Is the better outcome of HIV-2 infection linked to a preservation of memory B cells?

Results concerning this subject are described in Chapter 3.5.

In agreement with the Decreto-Lei 388/70, art. 8º, parágrafo 2, the results presented here were published, or are currently being prepared for publication, in the following scientific journals:

Rita Tendeiro[*], Rita Cavaleiro[*], Russell B. Foxall, Rui S. Soares, António P. Baptista, Perpétua Gomes, Emília Valadas, Rui M. M. Victorino, Ana E. Sousa.
Activation of blood monocytes and myeloid dendritic cells is a hallmark of HIV-2 infection.
Submitted for publication. [*]Equally contributed to this work.

Rita Tendeiro[*], Russell B. Foxall[*], António P. Baptista, Francisco Pinto, Rui S. Soares, Rita Cavaleiro, Emília Valadas, Perpétua Gomes, Rui M. M. Victorino, Ana E. Sousa.
PD-1 and its ligand PD-L1 are progressively up-regulated on CD4 and CD8 T-cells in HIV-2 infection irrespective of the presence of viremia.
AIDS 2012; 26(9): 1065-71. [*]Equally contributed to this work.

Rita Tendeiro, Adriana S. Albuquerque Russell B. Foxall, Rita Cavaleiro, Rui S. Soares, António P. Baptista, Maria V. D. Soares, Perpétua Gomes, Ana E. Sousa.
Preserved CD4 T-cell telomere length during long-lasting HIV-2 infection.
Submitted for publication.

Rita Tendeiro, Russell B. Foxall, Rui S. Soares, Emília Valadas, Perpétua Gomes, Rui M. M. Victorino, Ana E. Sousa.
Preserved naïve $CD4^+$ T-cell ability to proliferate upon IL-7 stimulation distinguishes HIV-2 from HIV-1 infection.
Manuscript in preparation.

Rita Tendeiro, Sofia Fernandes, Russell B. Foxall, José M. Marcelino, Nuno Taveira, Rui S. Soares, António P. Baptista, Rita Cavaleiro, Perpétua Gomes, Rui M. M. Victorino, Ana E. Sousa
Memory B-cell depletion is a feature of HIV-2 infection even in the absence of detectable viremia.
AIDS 2012, Epub ahead of print.

CHAPTER 3: RESULTS

3.1. Activation of blood monocytes and myeloid dendritic cells is a hallmark of HIV-2 infection

Submitted for publication:

Rita Tendeiro[*], Rita Cavaleiro[*], Russell B. Foxall, Rui S. Soares, António P. Baptista, Perpétua Gomes, Emília Valadas, Rui M. M. Victorino, Ana E. Sousa. **Activation of blood monocytes and myeloid dendritic cells is a hallmark of HIV-2 infection.**

[*]Equally contributed to this work.

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Abstract

HIV-2 represents a naturally-occurring form of attenuated HIV/AIDS, associated with low to undetectable plasma viral load throughout the course of disease, and a much slower rate of $CD4^+$ T-cell decline than HIV-1 infection. In spite of the high degree of structural homology between the two viruses, HIV-2 uniquely expresses Vpx, which was recently shown to overcome host restriction of HIV-1 replication in monocyte and myeloid dendritic cells (mDC). We investigated here, for the first time, monocyte and circulating mDC imbalances in HIV-2+ patients.

We found a significant expansion of $CD14^{bright}CD16^+$ monocytes, accompanied by up-regulation of HLA-DR expression and increased serum levels of soluble CD14 in HIV-2+ patients. In contrast to untreated HIV-1+ individuals, these alterations also occurred in early stage HIV-2 infection, before significant $CD4^+$ T-cell loss, and appeared to be unrelated to an increase in plasma LPS levels. Importantly, monocytes from HIV-2+ patients featured a marked up-regulation of the inhibitory molecules PD-L1/PD-1, which may contribute to the lack of significant increase of serum levels of pro-inflammatory cytokines and chemokines, typically produced by activated monocytes. Moreover, monocyte disturbances did not seem to translate into major mDC alterations in early stage HIV-2 infection. Nevertheless, HIV-2 disease progression, like untreated HIV-1 infection, led to mDC loss and acquisition of a more differentiated mDC phenotype.

In conclusion, our data provide evidence of a heightened state of monocyte activation during HIV-2 infection, which is accompanied by marked up-regulation of the inhibitory molecules PD-L1/PD-1, possibly contributing to the relatively “benign” course of HIV-2 disease. These results challenge the current paradigm regarding the role of monocytes in HIV/AIDS, and in inflammatory processes in general, and offer the chance to identify novel therapeutic targets.

Introduction

HIV infection leads to a state of heightened immune activation that is considered a main determinant of the progressive immunodeficiency [1].

Monocytes/macrophages and myeloid dendritic cells (mDC) are key producers of pro-inflammatory cytokines and chemokines, thereby directing activation and differentiation of adaptive immune responses, and recruiting T cells to sites of viral replication [2]. Recent data have highlighted the high degree of monocyte subset heterogeneity, each subset having particular phenotypic and functional properties, possibly generating distinct tissue macrophages [3-6]. In this respect, HIV-1 infection has been consistently associated with an expansion of $CD16^+$ monocytes, expressing variable levels of CD14 [7-10], which have been reported to be highly susceptible to infection and preferentially harbour the virus for long-term [11, 12]. Additionally, recent studies have confirmed that monocytes are able to differentiate into mDC at inflammatory sites [13], in agreement with previous *in vitro* data [14, 15]. These professional antigen-presenting cells (APC) have been shown to be depleted and functionally impaired in HIV-1 infection, particularly in its advanced stages and in individuals with high viremia [16, 17]. The mechanisms underlying monocyte and mDC disturbances during HIV-1 infection are not fully identified and are likely to be multifactorial [18].

These cells are particularly sensitive to stimulation by bacterial products through Toll-like receptor (TLR)-engagement [19, 20]. Chronically infected HIV-1+ individuals and simian immunodeficiency virus (SIV)+ rhesus macaques were reported to feature increased levels of circulating bacterial products, particularly lipopolysaccharide (LPS) [21, 22], a component of Gram-negative bacteria cell-walls that binds TLR4 [23]. This has been suggested to be related to a high level of gut microbial translocation resulting from the disruption of mucosal immunity associated with HIV-1 and SIV infections [24]. Although this has been highlighted as a main determinant of the hyper-immune activation that characterizes HIV disease [21], the relevance of microbial translocation in HIV/AIDS remains controversial, as some

African cohort studies featured no associations between circulating LPS and HIV disease progression [25, 26].

Both monocytes and mDC are targets for HIV infection as they express CD4, in addition to the two main HIV coreceptors, CCR5 and CXCR4 [27]. Nevertheless, these cells are unable to support productive HIV-1 infection, either *in vivo* or *in vitro* [28, 29]. Several mechanisms are likely to underlie the myeloid cell restriction of HIV-1 replication [30]. In this regard, intracellular host restriction factors have been intensively investigated [31, 32], particularly apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide like (APOBEC) [33] and SAM domain HD domain-containing protein 1 (SAMHD1) [34, 35]. Of note, Vpx, a viral protein uniquely encoded by HIV-2 and a group of SIV strains [36], is able to overcome SAMHD1-mediated lentiviral blockade by inducing its proteasomal degradation [35, 37].

HIV-2 infection is a naturally-occurring form of attenuated HIV disease, characterized by a slow rate of $CD4^+$ T-cell decline, undetectable to low levels of circulating virus and limited impact on the mortality of infected individuals [38]. This is despite similar levels of proviral DNA in HIV-2+ and HIV-1+ individuals, indicating a comparable number of infected cells and an equal ability of the two viruses to disseminate and establish viral reservoirs [39, 40]. The much lower levels of plasma viral load in HIV-2 than in HIV-1 infection is thought to account for the lower rates of horizontal and vertical transmission and the confinement of HIV-2 infection to West Africa, and related countries such as Portugal [41].

The reasons for the apparently better prognosis associated with HIV-2 infection remain elusive [42]. We have shown that chronic immune activation is a major driving force for $CD4^+$ T-cell depletion, in HIV-2 infection [43], as reported for HIV-1 [1]. Nevertheless, it is important to emphasize that the rate of progression of immune activation and the linked $CD4^+$ T-cell decline is much slower in HIV-2 than in HIV-1 disease [44].

There are very few data regarding mDC and monocytes in the context of HIV-2 infection. Duvall *et al* reported that mDC were less susceptible *in vitro* to HIV-2 than to

HIV-1 infection [45]. Moreover, although HIV-1 envelope proteins *per se* have been shown to induce mDC disturbances [46], we showed that the HIV-2 envelope protein did not alter the capacity of monocytes to differentiate into DC and the subsequent DC maturation [47]. This is despite our previous data showing that the HIV-2 envelope protein is able to induce TNF-α production by monocytes [48, 49] and to suppress T-cell proliferation through a monocyte-mediated mechanism [49].

Here, we provide the first comprehensive study of monocytes and mDC in untreated HIV-2+ and HIV-1+ individuals. Our data provide evidence of a heightened state of monocyte activation throughout the course of HIV-2 infection, which is accompanied by an up-regulation of the inhibitory molecules PD-L1/PD-1, possibly contributing to the relatively favourable outcome of HIV-2 infection.

Methods

Studied cohorts

The clinical and epidemiological characterization of the studied cohorts is shown in Table 1. All HIV-2+ (n=29) and HIV-1+ (n=22) individuals were antiretroviral therapy naïve, and had no ongoing opportunistic infections or tumours. The HIV cohorts were selected from patients under follow-up at the Hospital de Santa Maria, Lisbon, Portugal, in order to include individuals with comparable degrees of $CD4^+$ T-cell depletion (Table 1), although HIV-2+ were expected to be infected for significantly longer than HIV-1+ individuals [44]. In line with the comparable degree of $CD4^+$ T-cell depletion, both cohorts exhibited a similar up-regulation of activation markers within $CD4^+$ and $CD8^+$ T cells, as previously reported [43]. Importantly, 21 out of the 29 HIV-2-infected patients had undetectable viremia, and the highest level documented (26,263 RNA copies/ml) was significantly lower than the levels observed in HIV-1+ individuals (Table 1), as expected. Nevertheless, comparable levels of proviral DNA in PBMC were found (Table 1), in agreement with previous reports [39, 40]. Of note, the HIV-1 cohort included 4 individuals with undetectable viremia, formally defined as elite controllers, which usually represent less than 0.01% of the HIV-1+ individuals [50]. All individuals

gave written informed consent for blood sample collection and processing. The study was approved by the Ethical Board of the Faculty of Medicine, University of Lisbon.

Table 1. *Clinical and epidemiological data*[a].

	Seronegative	HIV-1	HIV-2
Numbers (male/female)	17 (6/11)	22 (17/5)	29 (9/20)
Age (years)	43±2 (27-57)	39±2 (23-61)[#]	48±3 (19-78)
Ethnicity (caucasian/other)	15/2	16/6	15/14
% $CD4^+$ T cells	44.9±2.0 (34.4-61.1)	22.8±3.3 (1.3-47.2)[***]	28.3±2.6 (7.1-54.1)[***]
$CD4^+$ T cells/µl	935±63 (518-1397)	569±105 (18-1848)[**]	648±79 (52-1511)[*]
% HLA-DR^+ (within CD4)	4.1±0.4 (1.9-7.6)[b]	17.1±3.0 (1.7-54.5)[***]	11.4±1.6 (1.9-36.3)[**]
% HLA-DR^+CD38^+ (within CD8)	4.4±1.4 (1.3-22.7)[b]	29.2±4.0 (1.4-62.2)[***]	20.3±3.5 (0.6-69.5)[**]
Viremia (RNA copies/ml)	NA	672,310±294,026 ($40-4.5x10^6$)[c; #]	2,937±1,090 (200-26,263)[c]
Proviral DNA (copies/10^6 PBMC)	NA	172±55 (5-975)	176±66 (5-1033)

[a] Data are mean±SEM with limits in brackets. NA, not applicable.
[b] n=16
[c] 4 of 22 HIV-1+ patients and 21 out of 29 HIV-2+ patients had undetectable viremia. In these cases, the cut-off value of the test (40 and 200 RNA copies/ml for HIV-1 and HIV-2, respectively) was used to calculate the mean.
[#] $p<0.05$ when comparing HIV-1 with HIV-2 infected patients; [*] $p<0.05$, [**] $p<0.01$, [***] $p<0.001$ when comparing infected cohorts with seronegative individuals.

Cell isolation and cell culture

PBMC were isolated from heparinised blood, immediately after venopuncture, by Ficoll-Hypaque density gradient centrifugation (Sigma, St. Louis, MO, USA). Monocytes were purified using magnetic beads (StemCell Technologies, France), with <2% T-cell contamination, as assessed by flow cytometry. *In vitro* responses to LPS were evaluated in total PBMC cultures, set at $2x10^6$ cells/ml in 24-well plates, in RPMI1640 (Gibco-Invitrogen, Paisley, U.K.) supplemented with 10% human AB serum

(Sigma-Aldrich, St Louis, MO, USA), 100 U/ml penicillin/100 μg/ml streptomycin (Gibco-Invitrogen) and 2 mM glutamine (Gibco-Invitrogen) in either the absence or presence of LPS (100 ng/ml; Sigma-Aldrich). After 22 hours of culture at 37 ºC and 5% CO_2, supernatant was harvested and stored at -80 ºC until subsequent cytokine/chemokine quantitation.

Flow cytometry

PBMC were surface stained as previously described [51] with the following anti-human mAbs (clone specified in brackets): FITC-conjugated HLA-DR (L243, BD Biosciences), CD16 (NKP15, BD Biosciences), CD20 (2H7, eBioscience), CD3 (CLB-T3/2) and CD14 (CLB-mon/1), both from Sanquin; PE-conjugated CD38 (HIT2, eBioscience), CD80 (L307.4, BD Biosciences), CD40 (5C3) and PD-L2 (MIH18), both from eBioscience; PerCP-conjugated CD4 (SK3, BD Biosciences), HLA-DR (L243, BD Biosciences); PE-Cy7-conjugated CD123 (6H6 , eBioscience) and CD14 (61D3, eBioscience); APC-conjugated CD8 (RPA-T8, eBioscience), CD11c (B-ly6) and CD86 (FUN-1) from BD Biosciences and PD-L1 (MIH1, eBioscience). At least 400,000 events were acquired in a large gate including lymphocytes (lymphogate) and monocytes (monogate), defined according to forward/side scatter characteristics. Within this gate, mDC were defined as lineage negative (lin^-; including FITC-conjugated mAbs against CD16, CD14, CD3, and CD20), $HLA\text{-}DR^+CD123^-CD11c^+$. Gated mDC were further analysed for the expression of PD-L1, PD-L2 and the co-stimulatory molecules CD40, CD80 and CD86. Monocytes were analysed within a monogate and further gated using CD14 and CD16 expression into four distinct subsets: $CD14^{dim}CD16^-$, $CD14^{bright}CD16^-$, $CD14^{bright}CD16^+$ and $CD14^{dim}CD16^+$. HLA-DR and PD-L1 expression was assessed using mean fluorescence intensity (MFI) within each subset. Lymphocytes were analysed within a lymphogate. Cells were acquired using a CANTO flow cytometer (BD Biosciences) and analysis was performed with FlowJo software (version 8.5.3, Tree Star, Inc., Ashland, OR). Absolute cell numbers in the blood were calculated by multiplying their representation by the absolute lymphocyte and/or monocyte counts obtained at the clinical laboratory.

Cytokines, chemokines, sCD14, and LBP and plasma LPS quantification

Human Cytokine LINCOplex (Millipore Corporation, Billerica, MA, USA) and the Luminex LX100 (Luminex Corporation, Austin, TX, USA) were used to quantify the following cytokines and chemokines according to manufacturer's instructions: IL-10, IL-12 p40, MIP-1β and TNF-α in culture supernatants collected as described above; and IL-10, IL-6, TNF-α, MCP-1 and MIP-1β levels in serum, obtained on centrifugation of freshly drawn blood and stored at -80ºC until use. ELISA tests were used to quantify serum levels of sCD14 (R&D systems, Minneapolis, MN, USA) and LBP (Hycult Biotechnologies, Uden, The Netherlands). Plasma LPS levels were quantified using the Limulus Amebocyte Assay (Cambrex, Walkersville, MD, USA) according to the manufacturer's instructions. Plasma samples were diluted and pre-treated as previously described [21]. All samples were assayed in duplicate.

Plasma viral load and proviral DNA assessment

HIV-1 viremia was quantified by RT-PCR (detection threshold of 40 HIV-1 RNA copies/ml, Roche, Basel, Switzerland). HIV-2 viremia was assessed using an RT-PCR-based assay [52] with a detection limit of 200 HIV-2 RNA copies/ml. The cut-off values of the tests were considered for statistical analysis in cases where detection was below these levels.

HIV-1 and HIV-2 total viral DNA (integrated and nonintegrated viral DNA species) were quantified using real-time PCR assays that amplify highly conserved regions in HIV-1 and HIV-2 *gag* with a detection range of 7 orders of magnitude and a sensitivity of 5 copies, as we have previously described [40].

Statistical analysis

Statistical analysis was conducted with Mann-Whitney tests and Spearman's correlations using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA).

Results were expressed as median and range, or mean±SEM, as indicated, and *P* values <0.05 were considered significant.

Results

HIV-2 infection is associated with marked monocyte activation and PD-L1 up-regulation

Monocyte imbalances were investigated in untreated HIV-2+ individuals (Table 1), who had low to undetectable plasma viral load, as expected [40], and were likely to have been infected for a long period given the very slow rate of $CD4^+$ T-cell decline that characterizes HIV-2 infection [44].

Numbers of circulating monocytes were similar in the untreated HIV-2 and HIV-1 cohorts, as compared to age-matched seronegative subjects (485±177, 485±133, and 496±251 cells/µl for HIV-2, HIV-1 and seronegative cohorts, respectively).

Monocytes were sub-divided into four subsets, defined according to the levels of CD14 and CD16 expression [6], as illustrated in Figure 1A: $CD14^{bright}CD16^{-}$, $CD14^{bright}CD16^{+}$, $CD14^{dim}CD16^{+}$ and $CD14^{dim}CD16^{-}$.

We found that the HIV-2 cohort featured a significant expansion of $CD14^{bright}CD16^{+}$ monocytes as compared to seronegatives (Figure 1A), that directly correlated with T-cell activation (Supplemental Figure 1A). This subset expressed the highest levels of HLA-DR, suggesting an activated phenotype [7] (Figure 1B). Notably, in comparison with seronegatives, the HIV-2 cohort showed a significant up-regulation of HLA-DR within whole monocytes and within each subset, with the exception of

$CD14^{dim}CD16^{-}$ cells. The same effect was observed in the $CD14^{dim}CD16^{+}$ subset of the HIV-2 cohort in comparison with HIV-1+ individuals (Figure 1B).

Additionally, HIV-2 infection was associated with an increase of the $CD14^{dim}CD16^{-}$ subset (Figure 1A), that lacked any apparent relationship with $CD4^{+}$ T-cell depletion or T-cell activation (Supplemental Figure 1B). The origin of the $CD14^{dim}CD16^{-}$ subset remains a subject of intense debate [53-56]. Although it may represent over-activated monocytes [57], possibly shedding CD14 at an increased rate [58], the levels of HLA-DR expression were not significantly higher in this population in the HIV cohorts than in seronegatives (Figure 1B). Conversely, a significant up-regulation of the inhibitory molecule PD-L1 within $CD14^{dim}CD16^{-}$ monocytes was observed in both infections, being significantly more marked in the HIV-2 cohort (Figure 1C).

PD-L1 expression was markedly up-regulated in HIV-2+ individuals, and this increase within the $CD14^{bright}CD16^{+}$ and $CD14^{dim}CD16^{-}$ subsets was statistically significant in comparison with HIV-1+ patients (Figure 1C). We also analyzed PD-1 expression levels (MFI) on total monocytes and found a significant increase in HIV-2+ patients (2098±216), both in comparison with HIV-1+ (1493±92, p=0.0272) and seronegative individuals (1405±167 and p=0.0174). In contrast, there were no significant differences between PD-1 levels in the HIV-1 and seronegative cohorts (p=0.4162). The levels of PD-1 and PD-L1 expression on monocytes were positively correlated in all three cohorts (HIV-2: r=0.6251, p=0.0004; HIV-1: r=0.5247, p=0.0122; seronegatives: r=0.7147, p=0.0019), suggesting that their expression may be co-coordinately regulated [59].

Finally, serum levels of soluble CD14 (sCD14), a classical marker of monocyte activation [58], were significantly higher in the HIV-2 cohort in comparison with seronegatives, reaching levels similar to those found in HIV-1+ individuals (Figure 1D).

Overall, our data provide evidence of a heightened state of monocyte activation during HIV-2 infection, which was accompanied by an up-regulation of the inhibitory molecules PD-L1/PD-1.

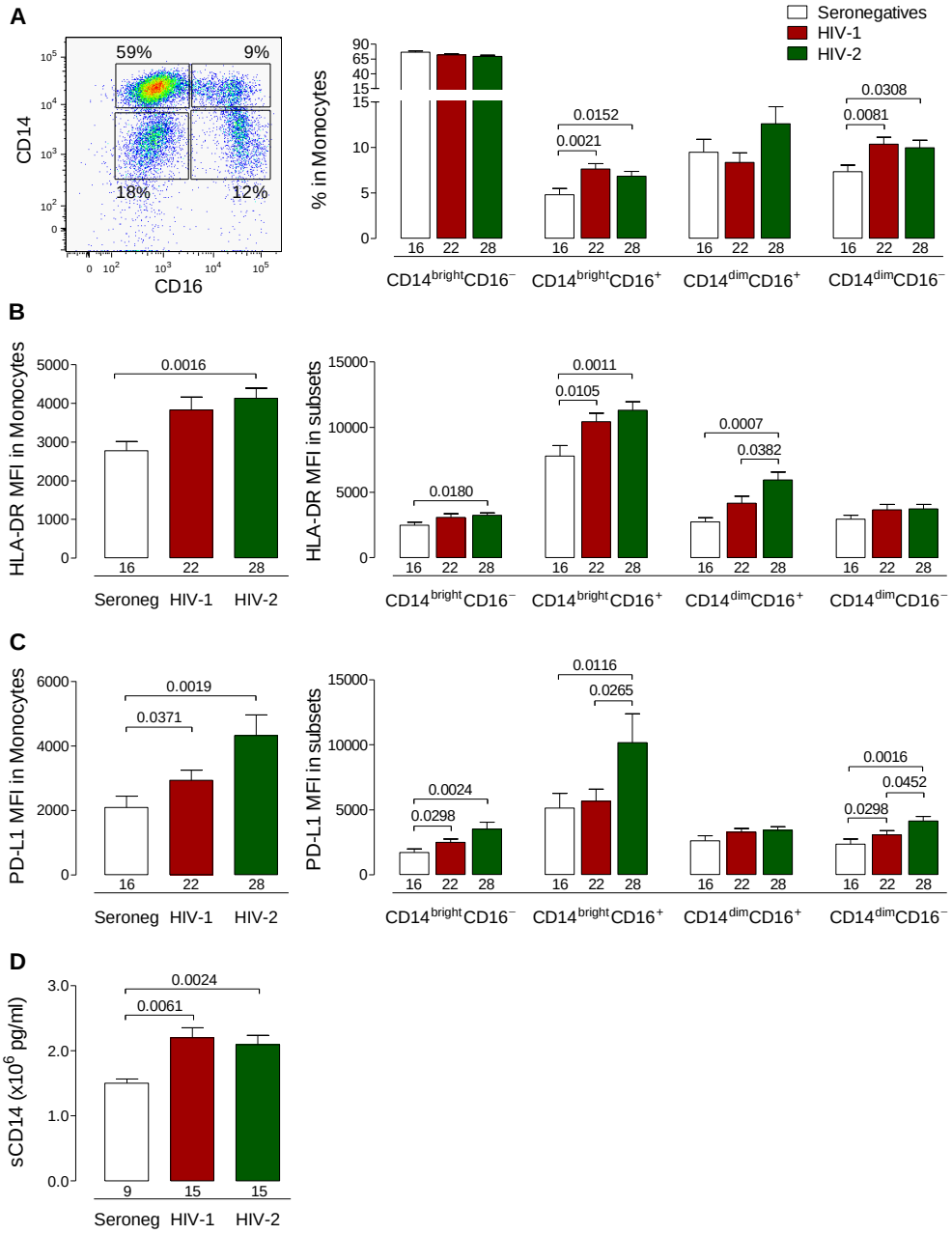

Figure 1. Monocyte imbalances in HIV-2-infected patients. (A) Representative flow cytometry analysis of monocyte subpopulations, the dot-plot refers to an HIV-2+ individual with 523 $CD4^+$ T cells/µl and undetectable levels of viremia. A gate including only monocytes (monogate) was defined by forward and side scatter characteristics. Cells were further gated according to the expression of CD14 and CD16. Four monocyte populations were defined, as follows: $CD14^{bright}CD16^-$, $CD14^{bright}CD16^+$, $CD14^{dim}CD16^+$ and $CD14^{dim}CD16^-$. Frequencies of the different subsets within the monogate are shown. The right-hand graph shows the relative proportion of each monocyte subset within total monocytes ($CD14^+$ cells) in HIV-1+ and HIV-2+ and seronegative (Seroneg) individuals. Expression of **(B)** HLA-DR and **(C)** PD-L1 in terms of Mean Fluorescence Intensity (MFI) on total monocytes (left-hand graph) and on monocyte subsets (right-hand graphs) in the three cohorts. **(D)** Serum sCD14 levels in subgroups of HIV-1+, HIV-2+ and seronegative individuals representative of the cohorts as a whole in terms of both $CD4^+$ T-cell counts and viremia (where applicable). Bars represent mean±SEM. Numbers under the bars represent the number of individuals analyzed. *p* values <0.05 were considered significant and are shown for all statistical differences observed.

Monocyte activation occurs in the absence of detectable viremia in HIV-2 infection

Next we assessed the relationship between the observed monocyte alterations and plasma viral load, since HIV-2 is associated with low to undetectable viremia irrespective of disease stage [43]. The expansion of $CD14^{bright}CD16^{+}$ monocytes was significantly higher in viremic patients despite the low levels of plasma HIV-2 load (200-26,263 RNA copies/ml), as shown in Figure 2A. Accordingly, the expansion of $CD14^{bright}CD16^{+}$ monocytes was directly correlated with viremia in HIV-2 infection ($r=0.3987$, $p=0.0356$) as well as in HIV-1 infection ($r=0.6117$, $p=0.0025$). Moreover, in contrast to HIV-1 infection, it was also positively correlated with the proviral DNA quantified in total PBMC in HIV-2+ individuals (HIV2: $r=0.5931$, $p=0.0018$, $n= 25$; HIV1: $r=0.1140$, $p=0.6338$, $n= 21$).

Nevertheless, aviremic HIV-2+ patients featured significantly increased expression of HLA-DR on the $CD14^{bright}CD16^{+}$ subset (Figure 2B), and of PD-L1 on both $CD14^{bright}CD16^{+}$ (Figure 2C) and $CD14^{dim}CD16^{-}$ subsets (Supplemental Figure 2), as well as increased sCD14 levels (Figure 2D), as compared to seronegatives, showing that monocyte activation occurred during HIV-2 infection, even in the absence of detectable circulating virus.

In contrast to HIV-2+ patients, who were largely aviremic irrespective of disease stage, only four HIV-1+ patients were able to control viral replication in the absence of antiretroviral therapy [50]. These four “elite controllers” had undetectable viremia for 2 to 10 years, and relatively well-preserved $CD4^{+}$ T-cell counts (814 ± 242cells/μl; range 344-1425). Notably, these four individuals lacked significant monocyte imbalances, except for a significant overrepresentation of the $CD14^{dim}CD16^{-}$ subset, which was also observed in aviremic HIV-2+ patients (Figure S2).

In conclusion, monocyte activation in HIV-2+ patients was largely unrelated to viremia, though circulating virus had a major impact on monocyte subset imbalances.

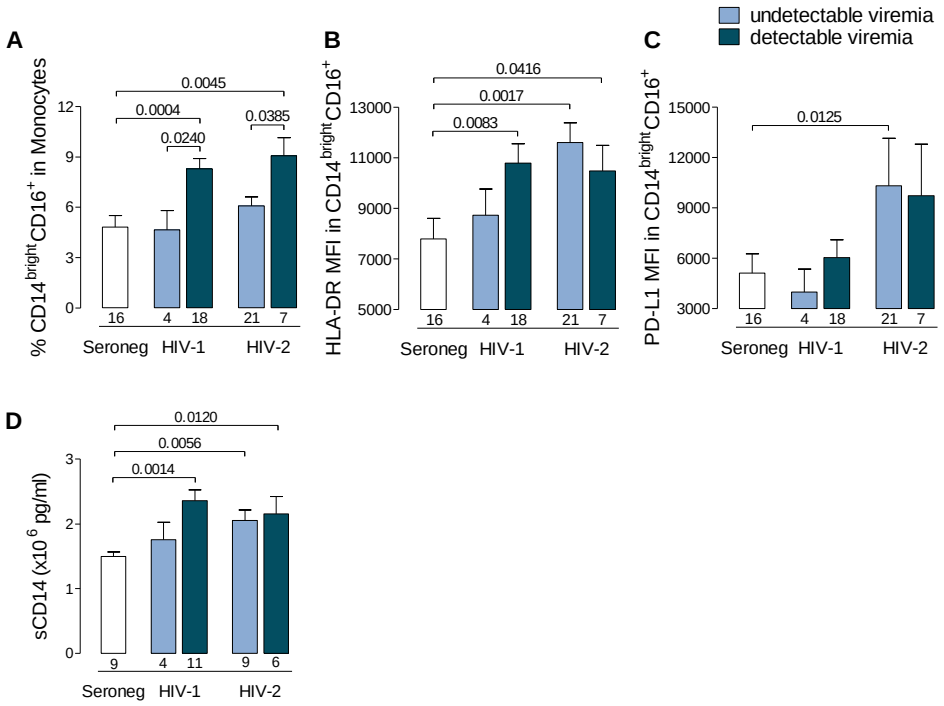

Figure 2. Impact of HIV-2 plasma viral load on monocyte imbalances. HIV-2 and HIV-1 cohorts were stratified according to presence or absence of detectable viremia and compared in terms of: frequency of $CD14^{bright}CD16^{+}$ cells within total monocytes (**A**); Mean Fluorescence Intensity (MFI) of HLA-DR **(B)** and PD-L1 **(C)** within the $CD14^{bright}CD16^{+}$ subset; and serum levels of sCD14 (**D**). Bars represent mean±SEM. Numbers under the bars represent the number of individuals analyzed. *p* values <0.05 were considered significant and are shown for all statistical differences observed. Seroneg: seronegative controls.

Monocyte activation occurs in early stages of HIV-2 infection

Importantly, as illustrated in Figure 3, the majority of monocyte disturbances in HIV-2 infection occurred before significant loss of $CD4^{+}$ T cells and up-regulation of T-cell activation markers [43]. Early stage HIV-2+ patients (>350 $CD4^{+}$ T cells/µl) showed a significant expansion of the $CD14^{bright}CD16^{+}$ subset (Figure 3A), accompanied by an over-expression of HLA-DR (Figure 3B) and a marked increase in the expression of PD-L1 (Figure 3C) in comparison with seronegative individuals. These alterations were not significant in their HIV-1+ counterparts, despite their higher viremia, and the comparable increases in sCD14 levels found in both infections (Figure 3D).

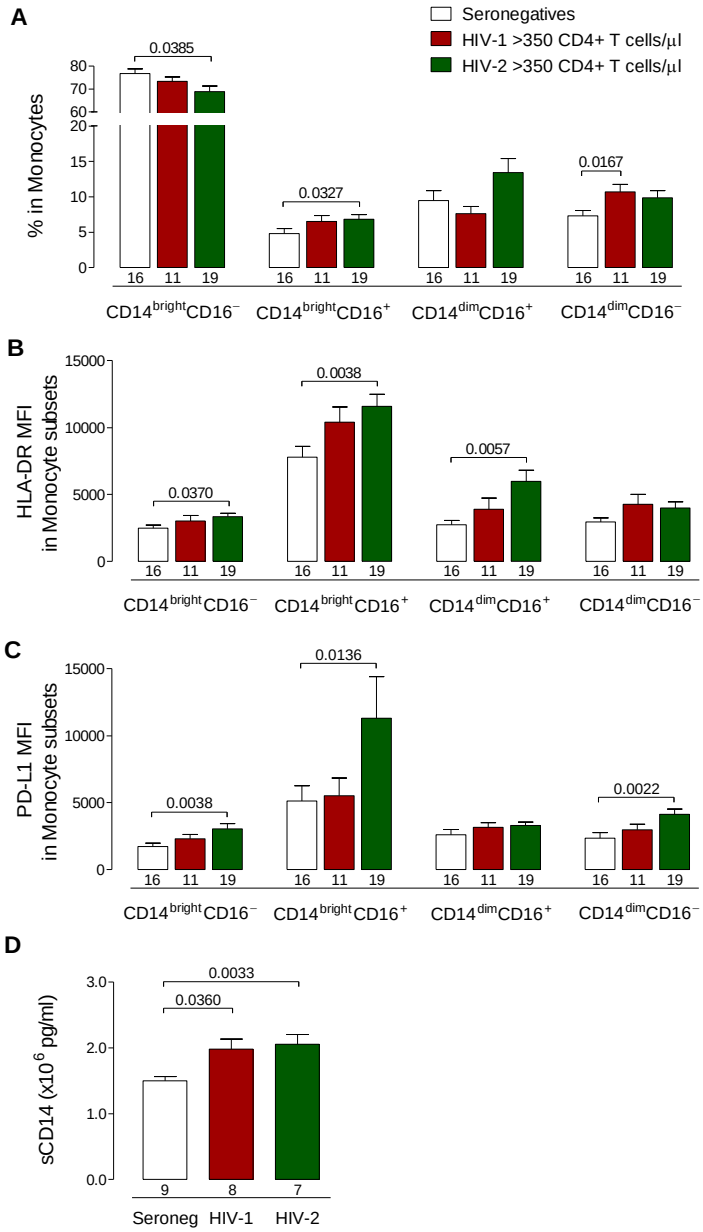

Figure 3. Monocyte imbalances in early stage HIV-2 disease. Comparison of HIV-2+ and HIV-1+ patients with >350 $CD4^+$ T cells/µl and seronegative (Seroneg) controls in terms of: (**A**) relative proportion of monocyte subsets defined according to the levels of expression of CD14 and CD16 within total monocytes ($CD14^+$ cells); (**B**) Mean Fluorescence Intensity (MFI) of HLA-DR within the different monocyte subsets; (**C**) Mean Fluorescence Intensity (MFI) of PD-L1 within the different monocyte subsets; and (**D**) serum levels of sCD14. Bars represent mean±SEM. Numbers under the bars represent the number of individuals analyzed. *p* values <0.05 were considered significant and are shown for all statistical differences observed.

Thus, monocyte disturbances were much more marked in early stage HIV-2 than HIV-1 infection. Given the recent studies suggesting a better ability of HIV-2 to

infect myeloid cells [34-36], we recruited additional HIV-2-infected patients, with early stage disease (>350 $CD4^+$ T cells/µl) and undetectable viremia, who showed a profile of monocyte disturbances similar to those above described, and sort-purified their monocytes to quantify the levels of proviral DNA. Importantly, proviral DNA levels were below the test cut-off (5 copies/10^6 cells) in all eight studied individuals, suggesting that the monocyte alterations were unrelated to a significant reservoir of infected monocytes in the blood.

Overall, monocyte activation and up-regulation of inhibitory molecules were features of HIV-2 infection from the early stage of disease.

Evidence against a major role of plasma LPS levels in HIV-2 infection

Circulating LPS, and other bacterial products arising from increased microbial translocation in the gut during HIV infection, have been suggested to play a key role in driving the generalized state of immune activation that characterizes HIV/AIDS pathogenesis and, particularly, monocyte activation [21, 22]. We quantified plasma LPS levels, and in agreement, analysis of the 3 combined cohorts revealed significant associations of plasma LPS levels with the degree of $CD4^+$ T-cell depletion (r=-0.3033, p=0.0244, n=55), the expression of HLA-DR on $CD4^+$ T cells (r=0.3056, p=0.0233, n=55), and the percentage of $CD38^+HLA\text{-}DR^+$ cells within $CD8^+$ T cells (r=0.3110, p=0.0208, n=55), as well as with sCD14 (r=0.3457, p=0.0488, n=33).

However, we found no statistically significant differences in plasma LPS levels between HIV-2+ and seronegative individuals (Figure 4A). Moreover, no statistical significant differences were found between plasma LPS levels in HIV-2+ patients with detectable and undetectable viremia (Figure 4A).

Conversely, HIV-1+ patients at a more advanced disease stage (<350 $CD4^+$ T cells/µl) featured significantly higher LPS levels than seronegatives (Figure 4A), though no significant correlations were found between plasma LPS levels and $CD4^+$ T-cell depletion or immune activation (p>0.05). Moreover, a significant association was

found in the HIV-1 cohort between higher plasma LPS levels and the relative loss of the $CD14^{bright}CD16^{-}$ population in favour of the other monocyte subsets, as shown in Figure 4B. No such association was seen in the HIV-2 cohort (Figure 4B). Additionally, plasma LPS levels positively correlated with the expression of HLA-DR on $CD14^{bright}CD16^{-}$ cells of HIV-1+ patients (r=0.4936, p=0.0230, n=21), which contrasts with the significant negative correlation found in the HIV-2 cohort (r=-0.5491, p=0.0183, n=18).

These results support a contribution of LPS in driving monocyte differentiation and activation *in vivo* during HIV-1 infection, but not in HIV-2 infection. Of note, no relationships were found between plasma LPS levels and PD-L1 expression (p>0.05).

We also quantified serum LPS-binding protein (LBP) that is known to be produced by gastrointestinal and hepatic epithelial cells upon LPS stimulation, and thought to be relevant for its interaction with CD14 [23]. No statistically significant differences were observed in LBP levels between HIV-2 and both HIV-1 and seronegative cohorts (Figure 4C), and there were no correlations between LBP levels and any of the aforementioned parameters (p>0.05).

Monocytes are known to become refractory upon repeated LPS stimulation [60]. Therefore, we compared the *ex vivo* ability of PBMC from HIV-2+ and HIV-1+ individuals to respond to LPS. A similar increase in IL-10, IL-12 p40, TNF-α and MIP-1β production was observed upon LPS stimulation in the three cohorts (Figure 4D). Moreover, there were no associations between cytokine production and markers of disease progression, except for the IL-10 stimulation index that was directly correlated with $CD4^{+}$ T-cell count (HIV-2: r=0.5286, p=0.0138, n=21; HIV-1: r=0.5250, p=0.0445, n=15). Of note, no relationship was found between the ability to produce cytokines upon LPS stimulation *in vitro* and plasma LPS (p>0.05).

In summary, plasma LPS did not appear to make a significant contribution to the monocyte imbalances we documented in HIV-2 infected patients.

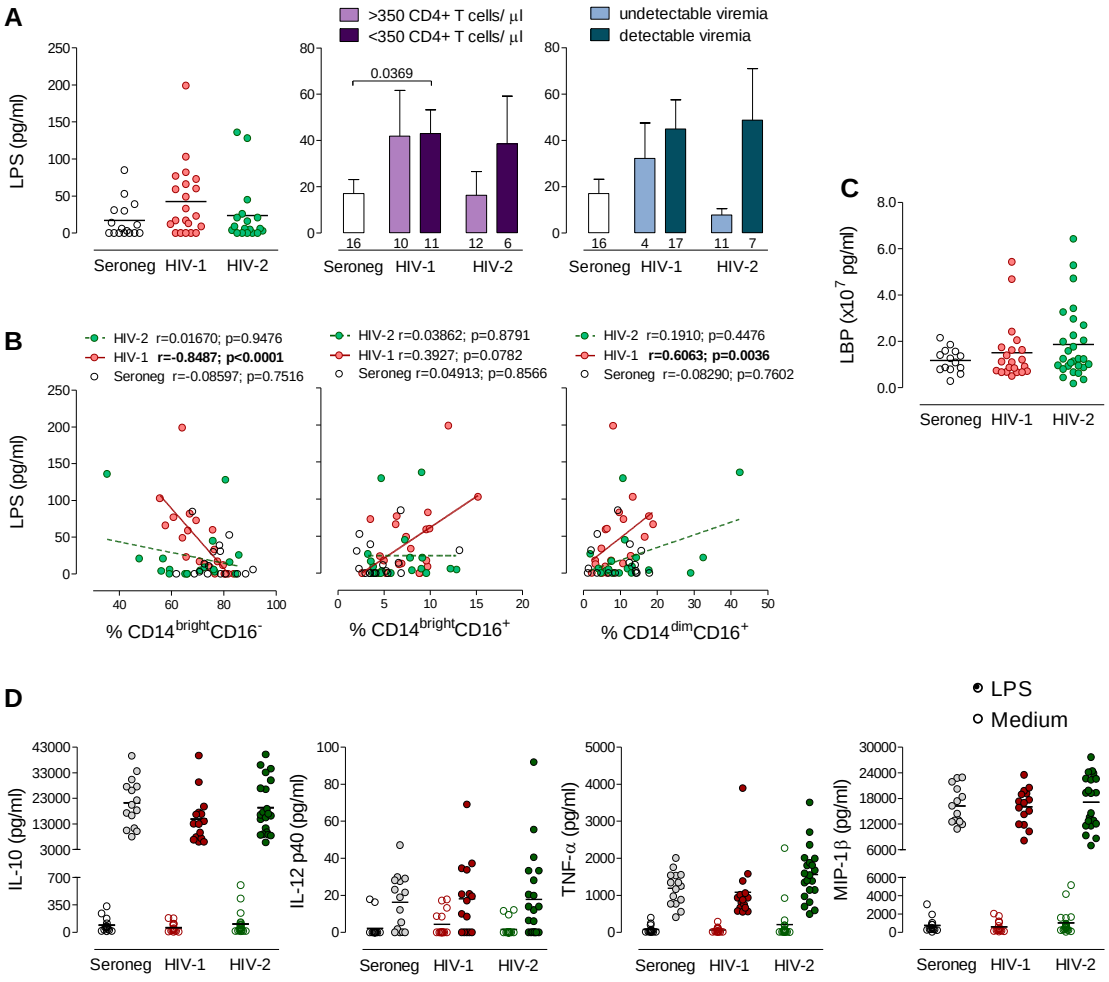

Figure 4. Plasma LPS during HIV-2 infection. (A) Plasma levels of LPS in HIV-1, HIV-2 and seronegative (Seroneg) cohorts (left). Each symbol represents one individual and bars represent mean. The infected cohorts were further stratified according to $CD4^+$ T-cell counts (>350 and <350 cells/µl; centre) and levels of viremia (undetectable *versus* detectable, right). Bars represent mean±SEM, with numbers under the bars representing the number of individuals analyzed. *p* values <0.05 were considered significant and are shown for all statistical differences observed. **(B)** Correlations between the levels of plasma LPS and the relative proportions of different monocytes subsets ($CD14^{bright}CD16^-$, $CD14^{bright}CD16^+$ and $CD14^{dim}CD16^+$) within total monocytes ($CD14^+$ cells). Values of $p<0.05$ were considered significant and are shown in bold. **(C)** Serum levels of LBP in the three cohorts. Each symbol represents one individual and bars represent mean. **(D)** Graphs show levels of IL-10, IL-12p40, TNF-α and MIP-1β in supernatants of freshly isolated PBMC cultured with LPS (closed circles) or medium alone (open circles) for 22 hours. Each symbol represents one individual and bars represent mean.

No major disturbances in serum cytokine and chemokine levels in early stage HIV-2 disease

We next assessed the serum levels of a panel of cytokines and chemokines known to be produced following monocyte stimulation: TNF-α, MIP-1β, MCP-1, IL-6, and IL-10.

Table 2. *Correlations between serum cytokine and chemokine levels with markers of disease progression, as well as with mDC and monocyte imbalances.*

	TNF-α	MIP-1β	MCP-1	IL-6	IL-10
% $CD4^+$ T cells	**-0.4026;0.011**	**0.4404;0.0057**	0.0814;0.6224	**0.3884;0.0145**	0.1029;0.5330
% HLA-DR^+ (CD4)	**0.5372;0.0004**	**-0.4624;0.0035**	-0.0466;0.7784	**-0.3452;0.0314**	0.0732;0.6578
% HLA-DR^+CD38^+ (CD8)	**0.5628;0.0002**	**-0.5205;0.0008**	-0.0648;0.6952	**-0.4206;0.0077**	0.0495;0.7648
Viremia (RNA cp/ml)	**0.4206;0.0207**	-0.1633;0.3886	0.0996;0.6007	-0.0220;0.9083	0.1547;0.4143
% $CD14^{bright}CD16^+$ (Mo)	**0.4267;0.0067**	**-0.3856;0.0168**	0.0978;0.5538	-0.3037;0.0602	0.2396;0.1418
HLA-DR MFI (Mo)	0.2320;0.1553	**-0.3876;0.0162**	-0.1557;0.3440	**-0.3523;0.0278**	-0.0574;0.7286
PD-L1 MFI (Mo)	0.1848;0.2600	-0.1493;0.3710	-0.0184;0.9114	-0.1831;0.2645	-0.2238;0.1709
Serum sCD14 (pg/ml)	**0.4194;0.0079**	-0.0080;0.9619	0.2582;0.1125	0.0535;0.7465	0.2896;0.0737
% mDC	**-0.5219;0.0007**	0.1827;0.2723	-0.0612;0.7114	0.2369;0.1464	**-0.4764;0.0022**
% $CD80^+$ (mDC)	**0.5350;0.0004**	-0.2403;0.1462	0.0683;0.6794	-0.2616;0.1076	0.2569;0.1143
% $CD86^+$ (mDC)	**0.3636;0.0229**	-0.2106;0.2043	-0.1063;0.5196	-0.3120;0.0531	0.2029;0.2154
% PD-$L1^+$ (mDC)	-0.0217;0.8959	**-0.3292;0.0436**	-0.1572;0.3392	**-0.370;0.0183**	-0.0377;0.8199

Analysis was performed with the whole cohorts combined (n=39; 15 HIV-2-infected patients, 15 HIV-1-infected patients and 9 seronegatives). Only infected cohorts (n=30) were considered for correlations with viremia. Mo: monocytes.
Spearman's correlation coefficient was used to assess the correlation between two variables. R;*p* values are shown. *p* values < 0.05 are highlighted in bold.

As shown in Table 2, serum TNF-α levels were directly correlated with $CD4^+$ T-cell depletion, viremia, and T-cell activation, as well as with the percentage of $CD14^{bright}CD16^+$ monocytes and sCD14 levels in the combined cohort (n=39; 9 seronegatives; 15 HIV-2+ and 15 HIV-1+ patients representative of the whole cohorts in terms of both $CD4^+$ T-cell counts and viremia). Nevertheless, TNF-α levels were not significantly higher in HIV-2+ than in seronegative individuals, in contrast to the HIV-1 cohort (Figure 5). Moreover, all HIV-2+ patients in early stage disease (>350 $CD4^+$ T cells/µl) had serum TNF-α levels within the range found in seronegatives (11.71±3.75 pg/ml; range: 3.2-28.49; n=7, for HIV-2+ *versus* 12.33±2.93 pg/ml; range: 5.08-28.16 pg/ml; n=9, for seronegatives, Supplemental Figure 3).

Conversely, HIV-2+ individuals featured significantly lower MIP-1β levels than seronegatives (Figure 5), though this decrease was not significant in early stage disease (Supplemental Figure 3). Notably, serum MIP-1β levels inversely correlated with $CD4^+$ T-cell depletion and T-cell activation in the combined cohort (Table 2).

No statistically significant differences were found regarding the levels of IL-6, MCP-1 and IL-10 in the three cohorts (Figure 5).

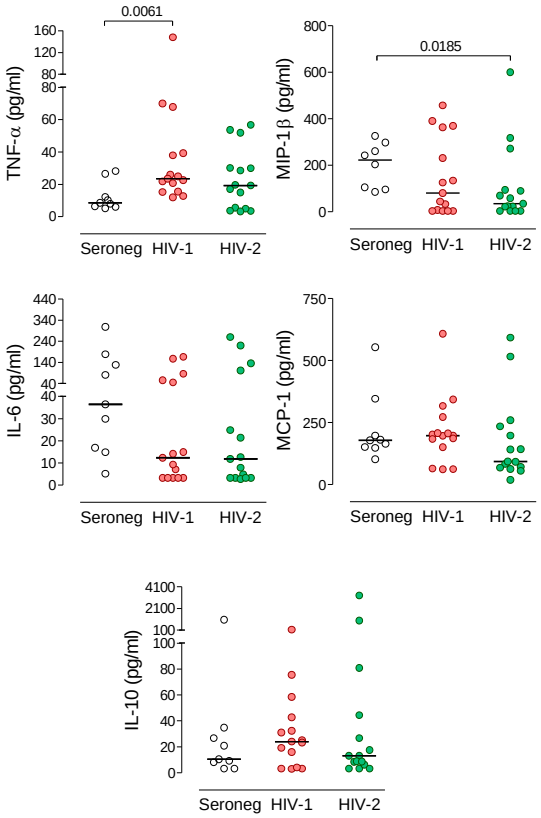

Figure 5. Serum cytokines and chemokines in HIV-2-infected patients. Serum levels of TNF-α, MIP-1β, IL-6, MCP-1 and IL-10 in subgroups of HIV-1+, HIV-2+ and seronegative (Seroneg) individuals representative of the cohorts as a whole in respect to both $CD4^+$ T-cell counts and viremia (where applicable). Each symbol represents one individual and bars represent median. *p* values <0.05 were considered significant and are shown for all statistical differences observed.

Importantly, there were no significant correlations between plasma LPS levels and the serum concentration of the cytokines and chemokines assessed (p>0.05). A recent report by Said *et al* has proposed that, in the context of HIV-1 infection, microbial translocation may up-regulate PD-1 levels on monocytes leading to increased IL-10 production upon PD-1/PD-L1 interaction [61]. However, no associations were found between PD-1 expression on monocytes and plasma LPS levels or serum IL-10 (p>0.05).

Overall, we showed that, despite the evidence of monocyte activation throughout HIV-2 infection, there were no major imbalances in the circulating levels of cytokines and chemokines typically produced by monocytes.

Similar alterations in circulating mDC in HIV-2 and HIV-1 infections

Monocytes differentiate into mDC both *in vitro* and *in vivo* [62, 63]. There are no data on circulating mDC during HIV-2 infection. It is plausible that the over-activation of monocytes could impact upon mDC.

The frequency of circulating mDC was assessed by flow cytometry, as illustrated in Figure 6A. HIV-2+ patients featured a statistically significant reduction in mDC levels as compared to seronegatives (Figure 6B), in direct correlation with the degree of $CD4^+$ T-cell depletion, as well as with the levels of $CD4^+$ and $CD8^+$ T-cell activation (Figure 6C). Moreover, the degree of mDC loss in HIV-2+ patients was similar to that found in HIV-1 infection (Figure 6B), in spite of the distinct viremia and the apparent impact of plasma viral load upon mDC levels. A statistically significant negative correlation between viremia and mDC levels was found in both infected cohorts (HIV-2: r=-0.5436, p=0.0028; HIV-1: r=-0.7073, p=0.0002). Moreover, as shown in Figure 6B, HIV-2+ patients with undetectable viremia, as well as the four HIV-1+ elite controllers, showed no decrease in mDC levels. On the other hand, similar levels of mDC depletion were found in viremic HIV-1+ and HIV-2+ patients, despite the much lower plasma viral load observed in HIV-2 infection, suggesting that even low levels of viremia may impact upon the mDC compartment (Figure 6B). Overall, viremia was strongly associated with loss of circulating mDC in both infections, contrasting with the more limited impact of viremia on the monocyte disturbances discussed above.

Of note, similar results were obtained when absolute mDC numbers were analysed (Supplemental Figure 4), confirming a marked mDC depletion in advanced HIV-2 disease, and relatively preserved circulating mDC numbers in early infection (>350 $CD4^+$ T cells/µl).

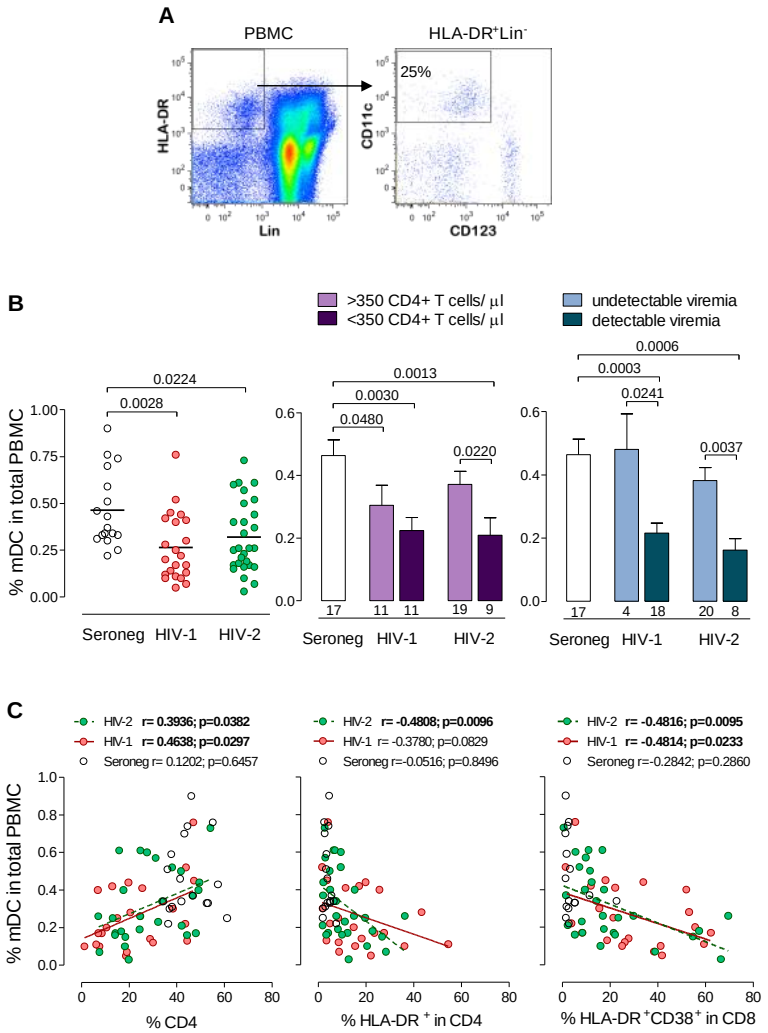

Figure 6. Circulating mDC in HIV-2-infected patients. (A) Representative flow cytometric analysis of mDC performed after successive gates on lymphocytes and monocytes defined according to forward and side scatter characteristics, as well as HLA-DR$^+$ cells that do not express lineage markers (left dot-plot); and CD123$^-$CD11c$^+$ cells (right dot-plot). Numbers represent the percentage of cells within the illustrated gates from an HIV-2+ patient with 523 CD4$^+$ T cells/µl and undetectable levels of viremia. The proportion of mDC within PBMC was 0.26%, which corresponded to 7 mDC/µl. **(B)** Circulating mDC levels in HIV-1, HIV-2 and seronegative (Seroneg) cohorts expressed as percentage within PBMC (left). Each symbol represents one individual and bars represent mean. The infected cohorts were further stratified in groups according to CD4$^+$ T-cell counts (>350 and <350 cells/µl, centre), or viremic and aviremic (below test cut-off, right), and mDC levels compared. Bars represent mean±SEM. Numbers under the bars represent the number of individuals analyzed. *p* values <0.05 were considered significant and are shown for all statistical differences observed. **(C)** Correlation between mDC frequency within PBMC and percentages of CD4$^+$ T cells (left), of HLA-DR$^+$ cells within CD4$^+$ T cells (centre), and of HLA-DR$^+$CD38$^+$ cells within CD8$^+$ T cells (right). *p* values <0.05 were considered significant and are shown in bold.

We then asked whether mDC maturation status was altered in HIV-2 infection, by assessing the expression levels of several maturation-associated molecules that are

related to the T-cell priming ability of mDC, namely the co-stimulatory molecules CD40, CD80 and CD86, and the inhibitory PD-1 ligands, PD-L1 and PD-L2.

Circulating mDC expressing CD40 and/or PD-L2 were almost absent in all cohorts (data not shown). CD80 expression was significantly higher in both HIV cohorts than in seronegatives (Figure 7A), and directly correlated with $CD4^+$ T-cell depletion and viremia (Table 3). CD86 expression was also increased in both infections but, in contrast to CD80, CD86 was significantly up-regulated even in early disease stages (Figure 7B), leading to an absence of significant correlation with $CD4^+$ T-cell depletion (Table 3). Of note, significant direct correlations between T-cell activation markers and expression of both CD80 and CD86 were seen in the HIV cohorts (Table 3). PD-L1 expression was also significantly increased throughout both infections (Figure 7C), and this up-regulation was also clear in aviremic HIV-2 individuals (Figure 7C). Interestingly, the PD-L1 MFI/CD86 MFI ratio within mDC was unaltered in the HIV-2 as compared to the seronegative cohort and was significantly higher in the HIV-2 than in the HIV-1 cohort (Figure 7D), further supporting a role for the PD-1/PD-L1 pathway in HIV-2 infection's relatively "benign" course. Accordingly, this ratio was significantly reduced in advanced stage HIV-1+ individuals as compared to seronegatives and their HIV-2+ counterparts (Figure 7D).

Table 3. *Correlations between mDC phenotype and parameters of disease progression.*

	Cohort	% $CD4^+$ T cells	Viremia (RNA cp/ml)	% $HLA\text{-}DR^+$ (CD4)	% $HLA\text{-}DR^+CD38^+$ (CD8)
$\%CD80^+$ (mDC)	Seroneg[a]	0.0147;0.9569	NA	0.1941;0.4713	-0.0882;0.7452
	HIV-1[b]	**-0.4778;0.0245**	**0.6029;0.0030**	-0.3954;0.0686	**0.5784;0.0048**
	HIV-2[c]	**-0.5485; 0.003**	**0.4427;0.0208**	**0.6014;0.0009**	**0.5614;0.0023**
$\%CD86^+$ (mDC)	Seroneg[a]	-0.0794;0.770	NA	0.0500;0.8541	-0.0823;0.7617
	HIV-1[b]	-0.2874;0.1947	**0.4258;0.0482**	**0.4591;0.0316**	**0.5144;0.0143**
	HIV-2[c]	-0.2519;0.2049	0.2971;0.1324	**0.4198;0.0293**	**0.4302;0.0251**
$\%PD\text{-}L1^+$ (mDC)	Seroneg[a]	0.0029;0.9914	NA	-0.0529;0.8456	-0.1618;0.5495
	HIV-1[b]	-0.1706;0.4479	0.0045;0.9840	0.2790;0.2086	-0.0056;0.9801
	HIV-2[c]	-0.0076;0.9699	-0.2947;0.1357	-0.0354;0.8608	-0.2033;0.3091

[a]n=16; [b]n=22; [c]n=27. Spearman's correlation coefficient was used to assess the correlation between two variables. R;*p* values are shown. *p* values <0.05 are highlighted in bold. Seroneg, seronegatives; NA, not applicable.

Of note, no correlation between plasma LPS levels and mDC levels and phenotype was seen in any cohort ($p>0.05$). However, when the three cohorts were analysed as a whole, the degree of mDC depletion was found to negatively correlate with serum IL-10 and directly with serum TNF-α levels (Table 2). The latter was also directly correlated with both CD80 and CD86 expression levels on mDC (Table 2).

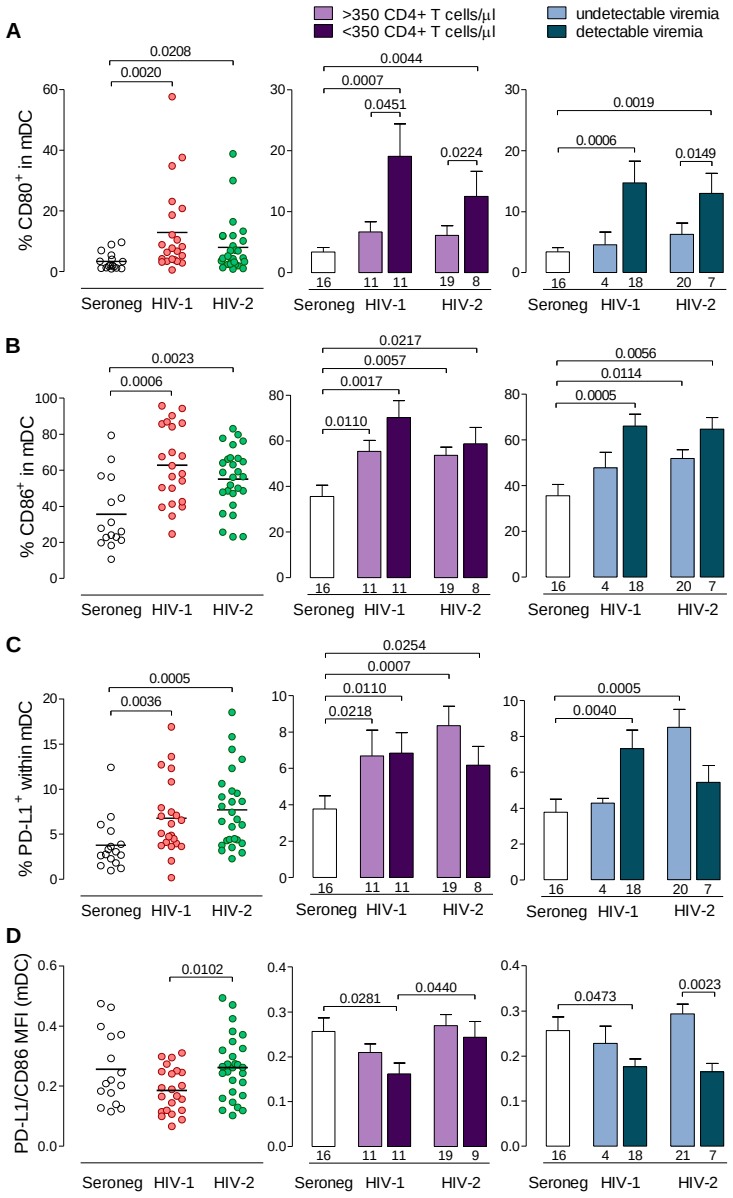

Figure 7. Expression of co-stimulatory and inhibitory molecules in circulating mDC in HIV-2 infection. Graphs show percentage of $CD80^+$ (**A**), $CD86^+$ (**B**) and $PD\text{-}L1^+$ (**C**) cells within mDC, as well as the ratio of the PD-L1 MFI and CD86 MFI within mDC (**D**), in HIV-1, HIV-2, and seronegative (Seroneg) cohorts (left). Each symbol represents one individual and bars represent mean. These analyses are also shown for the infected cohorts stratified according to $CD4^+$ T-cell counts (>350 and <350 cells/µl, centre) or levels of viremia (undetectable/detectable, right). Bars represent mean±SEM. Numbers under the bars represent the number of individuals analyzed. *p* values <0.05 were considered significant and are shown for all statistical differences observed.

In summary, these initial data regarding circulating mDC imbalances during HIV-2 infection revealed a progressive mDC loss associated with a more differentiated phenotype markedly similar to that observed during HIV-1 disease. Moreover, in early stage HIV-2 disease, the disturbances that we found in monocyte subsets did not seem to translate into major mDC alterations.

Discussion

This first study addressing monocyte and mDC disturbances during HIV-2 infection, revealed a heightened monocyte activation state and progressive mDC depletion in this natural model of attenuated HIV disease. We found that: 1) HIV-2+ patients featured a significant expansion of $CD14^{bright}CD16^{+}$ monocytes accompanied by up-regulation of HLA-DR expression and increased levels of serum sCD14; 2) monocyte activation occurred even in HIV-2+ individuals with undetectable viremia; 3) unlike HIV-1 infection, monocyte alterations were present from early stage HIV-2 disease; 4) monocyte imbalances were neither associated with increased plasma LPS or LBP levels, nor impairments in their ability to respond to LPS upon *in vitro* stimulation; 5) HIV-2+ patients featured an over-expression of the inhibitory molecules PD-1/PD-L1 in myeloid cells that may contribute to the lack of significant increase in serum levels of pro-inflammatory cytokines and chemokines usually produced by activated monocytes; 6) despite the reduced viremia, HIV-2, like HIV-1 infection, was associated with a progressive decline of circulating mDC levels and acquisition of a more differentiated mDC phenotype.

This heightened state of monocyte activation could be related to the proposed better ability of HIV-2 to infect myeloid cells [29]. Myeloid cell restriction of HIV-1 replication has been recently suggested to be mainly due to the effects of SAMHD1 [34, 35]. This molecule is targeted by Vpx, a protein only encoded by HIV-2 and some SIV strains, but not HIV-1 [64]. Thus, HIV-2 would be expected to circumvent SAMHD1-imposed restriction to viral replication in monocytes. Additionally, $CD16^{+}$ monocytes (expressing variable levels of CD14), a population that we found to be expanded in

HIV-2+ patients, have been shown to have increased susceptibility to HIV-1 infection [11, 12]. However, we found no detectable proviral load in monocytes isolated from HIV-2+ individuals in early-stage disease. These data indicate that, whilst Vpx can facilitate monocyte infection by HIV-2, additional restriction pathways are probably activated upon HIV-2 infection of these cells. These results argue against a significant amount of productive HIV-2 replication in monocytes. Nevertheless, it is possible that viral entry *per se* impacts on monocyte activation, a possibility that deserves future exploration in *in vitro* studies.

Increased translocation of microbial products, such as LPS, at the intestinal mucosa has been proposed to contribute to monocyte activation and correlate with disease severity during HIV infection [21, 22]. In agreement, our data suggest that LPS has a significant impact on monocyte activation and differentiation in HIV-1+ individuals. However, we found no association between monocyte and mDC disturbances with circulating LPS in HIV-2 infection. Higher levels of monocyte activation were found in HIV-2+ patients as compared to HIV-1+ counterparts, as illustrated by HLA-DR expression levels. These findings argue against a major role of microbial translocation in driving immune activation and disease progression in HIV-2 infection, similar to the situation reported for several African cohorts [25, 26]. Of note, even though our HIV-2 cohort was enriched in individuals of African origin, no associations were found between ethnicity and LPS levels that could impact on our results (data not shown).

The ability of monocytes to continuously replenish tissue macrophages and dendritic cells is thought to be important in the homeostasis of these populations, particularly in the context of inflammation [63]. Despite the overall maintenance of monocyte numbers observed during HIV-2 and HIV-1 infection, mDC were markedly reduced, particularly in more advanced disease stages.

This is the first report of circulating mDC depletion during HIV-2 infection. The underlying mechanisms are likely to be multiple. Our finding of a direct association between T-cell activation and mDC disturbances in both HIV-1 and HIV-2 infections, support a major contribution of persistent immune activation. Although it has been

suggested that disturbances in cell-trafficking may contribute to a preferential loss of circulating mDC in HIV-1 infection, the limited data available on lymphoid tissues from HIV-2+ individuals precluded the evaluation of this possibility [65, 66]. Additionally, mDC could be depleted by direct virus infection, as suggested by recent studies addressing the role of Vpx [34, 35]. Although this possibility was not addressed in our study, previous data have shown that mDC are less susceptible to HIV-2 than to HIV-1 infection *in vitro* [45]. Our data also suggest that factors other than direct viral infection are implicated in the mDC decline, as we found a similar decrease in mDC levels in HIV-2+ and HIV-1+ individuals, despite their distinct levels of viremia. Another explanation that warrants consideration for the documented loss of circulating mDC in HIV-2+ patients would be decreased bone-marrow precursor production of mDC, particularly given our previous observation of a marked combined leukopenia and neutropenia in HIV-2+ patients (AE Sousa, unpublished data). Moreover, it is recognized that HIV-1 envelope (Env) protein interferes with the differentiation of monocytes into DC *in vitro* [67]. However, we have shown that HIV-2 envelope glycoprotein gp105 does not impact upon this process [47], which argues against this as a major contributing factor to the depletion of circulating mDC in HIV-2+ patients.

The hyper-activated state that characterized both monocytes and mDC was, in HIV-2 disease, associated with a marked up-regulation of inhibitory molecules involved in limiting exacerbated immune activation (PD-1/PD-L1) [68]. Of note, this was not observed in the few HIV-1 “elite controllers” included in this study, although this should be investigated in larger cohorts of these particular HIV-1+ individuals that, similarly to HIV-2+ patients, have low levels of viremia without antiretroviral drugs.

HIV-2+ patients featured a significantly higher ratio of inhibitory (PD-L1) and co-stimulatory (CD86) molecules on mDC, suggesting that overall balance of signaling is tipped towards suppression, which may in turn help limit the rate of increase in immune activation. A recent report has suggested that, in the context of HIV-1 infection, microbial translocation may up-regulate PD-1 levels on monocytes, leading to increased IL-10 production upon PD-L1 binding, with consequent attenuation of immune responses [61]. This was unlikely to occur in the context of HIV-2 infection

since we found no evidence of a significant contribution of microbial translocation to the observed monocyte and mDC disturbances.

Of note, high levels of PD-L1 up-regulation were observed even in the absence of detectable viremia and/or significant $CD4^+$ T-cell loss, suggesting that, in HIV-2 infection, the PD-1/PD-L1 pathway may be limiting immune activation from the earliest stages of disease. This was further supported by the lack of any significant alterations in monocyte-produced pro-inflammatory cytokine and chemokines in the serum of HIV-2+ individuals. Our observations are in agreement with recently published data suggesting that, during HIV-2 disease, there is a strict control of PD-1/PD-L1 expression on T cells, which is disrupted in the context of HIV-1 infection, possibly helping limit immunopathology and restrain disease progression [59]. Additionally, our previous data on plasmacytoid dendritic cells (pDC) during HIV-2 disease have also showed significantly increased levels of PD-L1 expression, irrespective of disease stage [51]. Taken together, these data suggest that generalized PD-L1 over-expression may constitute a specific “HIV-2 signature”.

In conclusion, we investigated here, for the first time, the monocyte and mDC compartments during HIV-2 infection, a naturally-occurring form of attenuated HIV disease. We found evidence of monocyte activation since early stage HIV-2 disease, and a progressive depletion of circulating mDC with up-regulation of activation markers, tightly correlated with markers of T-cell activation. Our data emphasized the relationship between generalized immune activation and myeloid cell disturbances in HIV-2+ individuals, albeit with no apparent link to microbial translocation. Moreover, monocyte and mDC featured marked up-regulation of the inhibitory molecule PD-L1 from early on in HIV-2 infection, possibly helping limit activation-related immune-pathology and resulting in a more “benign” disease course. These findings challenge current paradigms of the role of monocytes and mDC in HIV/AIDS and have the potential to identify novel strategies to modulate inflammatory states.

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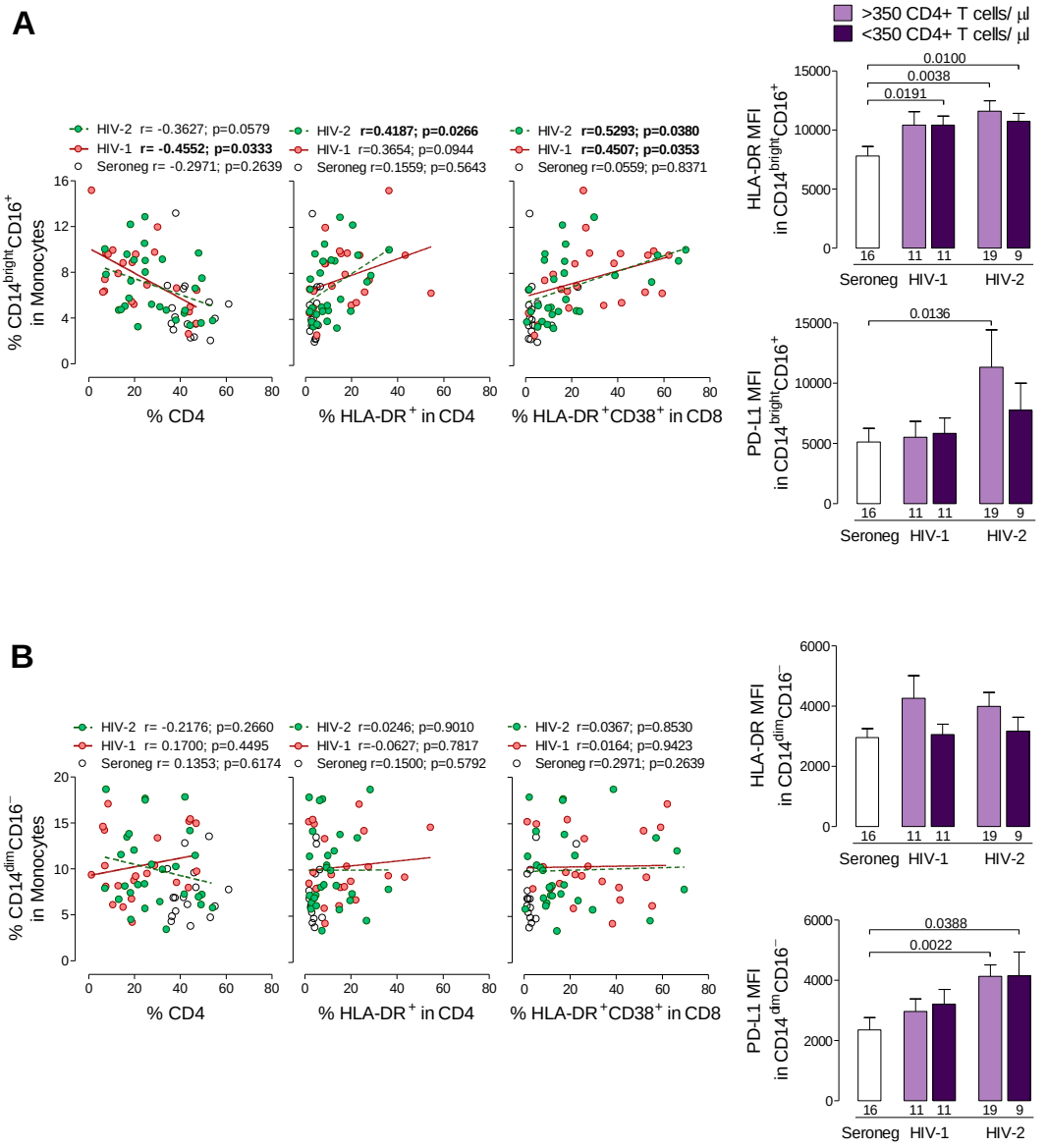
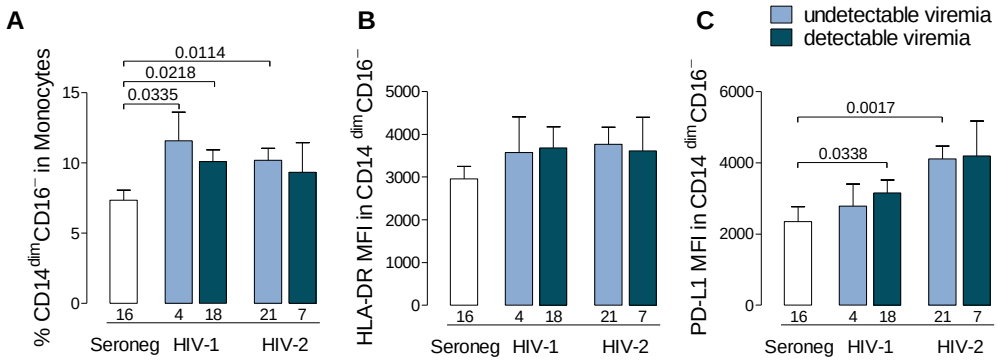
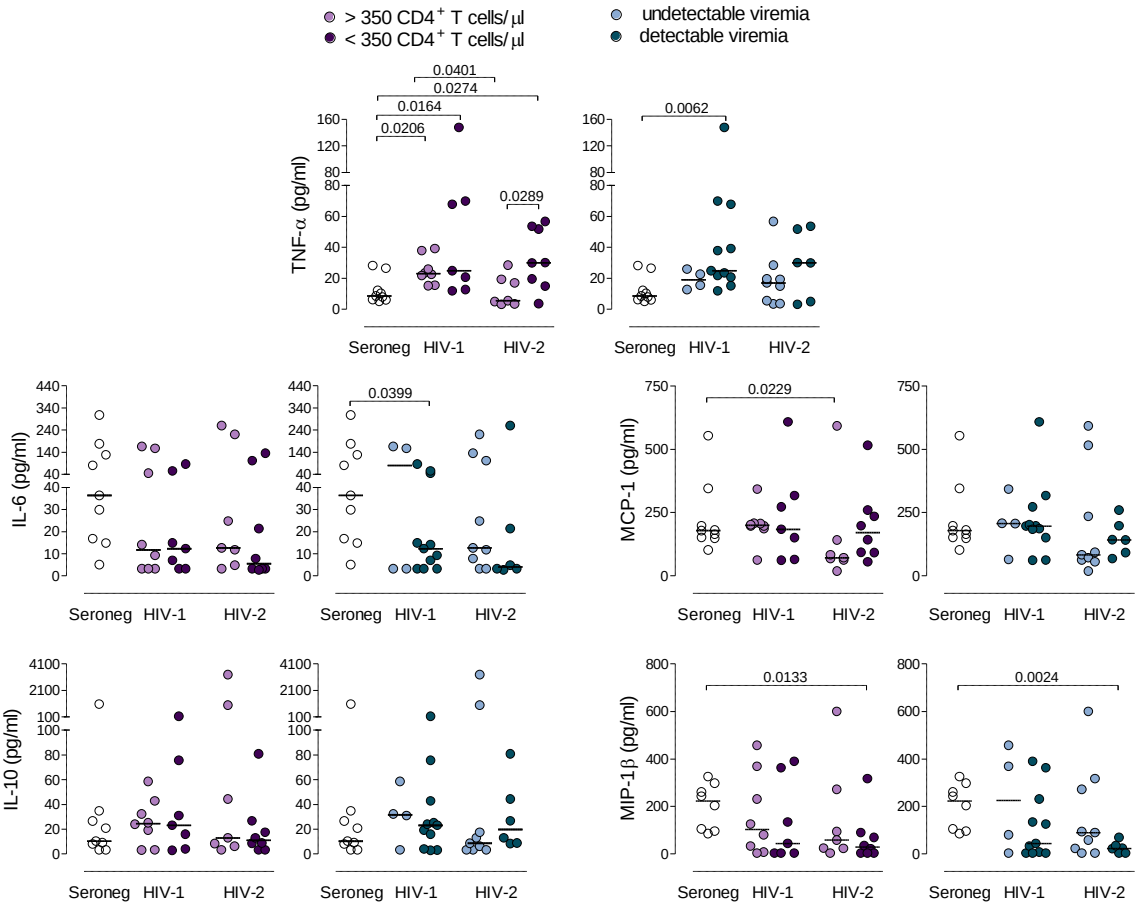
Supplemental Data



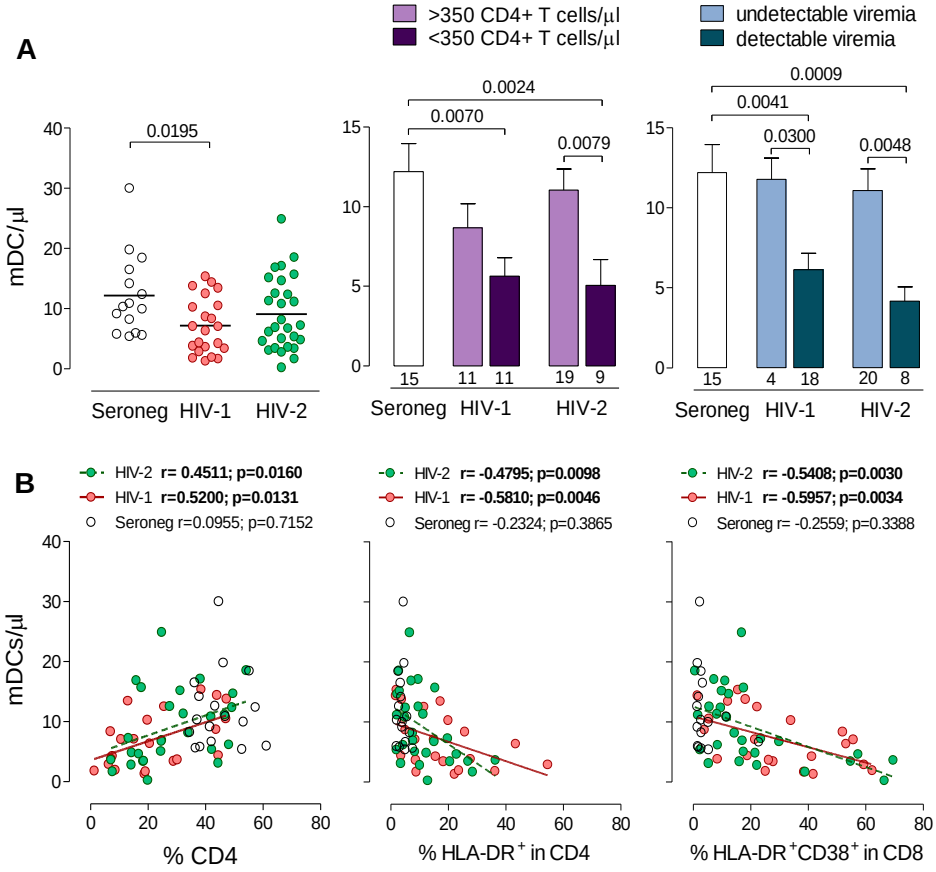
Supplemental Figure 1. Relationship between imbalances in CD14^{bright}CD16^{+} and CD14^{dim}CD16^{-} subsets and degree of CD4^{+} T-cell depletion or levels of T-cell activation. (A) Correlation between proportion of CD14^{bright}CD16^{+} cells within total monocytes (CD14^{+}) and percentages of CD4^{+} T cells, of HLA-DR^{+} cells within CD4^{+} T cells, and of HLA-DR^{+}CD38^{+} cells within CD8^{+} T cells in HIV-2+, HIV-1+ and seronegative (Seroneg) individuals; levels of expression of HLA-DR (top-right) and PD-L1 (bottom-right) within this subset in HIV infected cohorts stratified according to CD4^{+} T-cell counts (>350 and <350 cells/µl) and seronegatives. **(B)** The same analysis regarding the CD14^{dim}CD16^{-} subset. *p* values <0.05 were considered significant and are shown for all statistical differences observed. Bars represent mean±SEM. Numbers under the bars represent the number of individuals analyzed.



Supplemental Figure 2. Impact of HIV-2 plasma viral load on the imbalances of the CD14^{dim}CD16^{-} monocyte subset. HIV-2 and HIV-1 cohorts were stratified according to presence or absence of detectable viremia and compared in terms of: (**A**) frequency of CD14^{dim}CD16^{-} cells within total monocytes (CD14^{+} cells); and Mean Fluorescence Intensity (MFI) of HLA-DR (**B**) and PD-L1 (**C**) within the CD14^{dim}CD16^{-} subset. Bars represent mean±SEM. Numbers under the bars represent the number of individuals analyzed. *p* values <0.05 were considered significant and are shown for all statistical differences observed. Seroneg: seronegative controls.



Supplemental Figure 3. Serum cytokines and chemokines in HIV-2 infected patients according to CD4$^+$ T-cell depletion and viremia status. Graphs show the levels of TNF-α, IL-6, IL-10, MCP-1 and MIP-1β in the serum of HIV-1+, HIV-2+ and seronegative (Seroneg) individuals, that were representative of the whole cohorts in respect to both CD4$^+$ T-cell counts and viremia (where applicable). Each symbol represents one individual and bars represent median. The infected cohorts were stratified according to CD4$^+$ T-cell counts (>350 and <350 cells/µl, left-side graph) or undetectable/detectable levels of viremia (right-side graph). *p* values <0.05 were considered significant and are shown for all statistical differences observed.



Supplemental Figure 4. Circulating mDC in HIV-2-infected patients. (A) Graph on the left shows absolute mDC numbers in HIV-1 and HIV-2 cohorts and seronegative (Seroneg) individuals. Each symbol represents one individual and bars represent mean. The infected cohorts were stratified, either according to $CD4^+$ T-cell counts (>350 and <350 cells/µl), or presence or absence of viremia (below test cut-off), and mDC counts compared. Bars represent mean±SEM. Numbers under the bars represent the number of individuals analyzed. *p* values <0.05 were considered significant and are shown for all statistical differences observed. **(B)** Correlation between mDC numbers and percentages of $CD4^+$ T cells (left), of $HLA\text{-}DR^+$ cells within $CD4^+$ T cells (centre), and of $HLA\text{-}DR^+CD38^+$ cells within $CD8^+$ T cells (right). *p* values <0.05 were considered significant and are shown in bold.

3.2. PD-1 and its ligand PD-L1 are progressively up-regulated on $CD4^+$ and $CD8^+$ T cells in HIV-2 infection irrespective of the presence of viremia

Publication:

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[*]Equally contributed to this work.

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Abstract

Objective: Hyper-immune activation is a main determinant of HIV disease progression, potentially counteracted by T-cell inhibitory pathways. Here we investigated, for the first time, inhibitory molecules in HIV-2 infection, a naturally occurring attenuated form of HIV disease, associated with reduced viremia and very slow rates of $CD4^+$ T-cell decline.

Design: PD-1/PD-L1, an important pathway in limiting immunopathology, and its possible relationship with TIM-3, a recently identified inhibitory molecule, were studied in untreated HIV-2 and HIV-1 cohorts, matched for degree of $CD4^+$ T-cell depletion, and non-infected individuals.

Methods: Flow cytometric analysis of T-cell expression of PD-1, PD-L1 and TIM-3, combined with markers of cell differentiation, activation, cycling and survival. Statistical analysis performed using ANOVA, Mann-Whitney/Wilcoxon tests, Spearman's correlations, multiple linear regressions and canonical correlation analysis.

Results: T-cell expression of PD-1 and PD-L1 was tightly associated and directly correlated with $CD4^+$ T-cell depletion and immune activation in HIV-2 infection. No such correlation was found for PD-L1 expression in HIV-1+ patients. Central-memory and intermediate memory cells, rather than terminally-differentiated T cells expressed the highest levels of both PD-1 and PD-L1 molecules. Conversely, TIM-3 expression was independent of T-cell differentiation and dissociated from cell-cycling, suggesting distinct induction mechanisms. Importantly, in contrast with HIV-1, no significant increases in TIM-3 expression were found in the HIV-2 cohort.

Conclusions: Our data suggest that PD-1/PD-L1 molecules, rather than markers of T-cell exhaustion, may act as modulators of T-cell immune activation, contributing to the slower course of HIV-2 infection. These data have implications for the design of ART-complementary immune-based strategies.

Introduction

The hallmark of HIV immunopathogenesis is a hyper-immune activated state that directly correlates with progression to AIDS [1, 2]. Since the process of T-cell activation is tightly regulated, resulting from a balance between stimulatory and inhibitory signaling pathways [3], it is plausible that inhibitory molecules play a major role during HIV infection. The Programmed Death-1 (PD-1) receptor/ligand network is considered one of the main pathways capable of limiting the deleterious effects of inappropriate/exacerbated immune activation [4]. In the context of HIV-1 infection, PD-1 was first described as a cellular exhaustion marker for HIV-specific T cells, being associated with reduced proliferation and cytokine secretion capacities, and its blockade leading to partial recovery of these functional deficits [5-7]. Recently, however, several groups have suggested that increased PD-1 levels may instead limit tissue damage resulting from chronic immune activation [8, 9] and/or persistent antigenemia associated with ongoing viral replication [10-12]. Furthermore, an increasing body of evidence indicates that PD-1 expression alone, may not always be associated with, nor sufficient to characterize an exhausted phenotype, with the co-expression of other inhibitory molecules, like the Ig superfamily member T-cell immunoglobulin and mucin-domain containing molecule-3 (TIM-3), being essential to define T-cell exhaustion [13-18].

Here we sought to investigate the contribution of the PD-1/PD-L1 pathway and TIM-3 to HIV/AIDS immunopathogenesis through the study of HIV-2 infection, a naturally-occurring form of attenuated HIV disease [19]. HIV-2+ individuals typically feature lower rates of $CD4^+$ T-cell depletion, reduced amounts of circulating virus and slower disease progression as compared to HIV-1+ patients [20]. Nevertheless, when matched for the degree of $CD4^+$ T-cell depletion, both infections feature the same levels of immune activation [2, 21]. Data regarding the expression of PD-1, PD-L1 and TIM-3 in HIV-2+ patients are extremely limited [11]. Investigating the contribution of inhibitory pathways to the slower progression to AIDS observed in HIV-2 infection may provide a rationale for novel therapeutic interventions in HIV/AIDS.

Methods

Study populations

The study included 28 HIV-2+ and 22 HIV-1+ antiretroviral therapy (ART)-naïve, asymptomatic individuals and 16 seronegative controls (Supplemental Table 1 for clinical and epidemiological data). Written consent for blood sampling and processing was provided, and the study approved by the Ethical Board of the Faculty of Medicine of the University of Lisbon.

Flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinised blood and surface and/or intracellularly stained, as previously described [22, 23]. At least 100,000 events were acquired with a FACSCanto. Analysis was performed using a "fluorescence minus one" strategy with FlowJo software.

Plasma viral load assessment

Viremia was quantified by reverse transcriptase PCR (RT-PCR). Detection thresholds were: 40 RNA copies/ml for HIV-1 (Roche, Basel, Switzerland), and 200 RNA copies/ml for HIV-2, using a previously described in-house assay [24].

Statistical analysis

Statistical analysis was performed using GraphPad Prism v5.00, SPSS v15.0 and MATLAB v7.3. Data were compared using one-way or repeated measures ANOVA (with the Dunns post-test), followed by Mann-Whitney or Wilcoxon tests, as appropriate; associations were assessed by Spearman's correlation coefficient, multiple linear regressions and canonical correlation analysis. p-values <0.05 were considered significant.

Results

PD-1 expression on T cells

We compared ART-naïve HIV-2 and HIV-1 cohorts matched for $CD4^+$ T-cell depletion, with similar levels of T-cell activation, but distinct viremia (Supplemental Table 1). PD-1 expression tended to be higher on both $CD4^+$ and $CD8^+$ T cells in HIV cohorts as compared to seronegatives, though HIV-2 featured significantly lower levels within $CD8^+$ T cells than HIV-1 (Figure 1A). Of note, similarly to seronegatives, HIV-2+ individuals exhibited a significant correlation between PD-1 expression on $CD8^+$ and $CD4^+$ T cells, which was not found in the HIV-1 cohort (Supplemental Figure 1).

Next, we investigated whether increased PD-1 expression was related to HIV-associated T-cell imbalances [25-27]. PD-1 expression varied with $CD8^+$ T-cell differentiation in HIV-2+ in a similar way to that previously described for HIV-1+ and seronegative individuals [28, 29]. PD-1 levels were lowest on naïve cells, and peaked at the $CD45RO^+CD27^+$ stage (Supplemental Figure 1), supporting the view that PD-1 up-regulation, rather than being related to terminal T-cell differentiation and exhaustion, may indicate T-cell activation upon antigen encounter. Whilst PD-1 expression was significantly increased on all $CD8^+$ T-cell subsets in HIV-1+ relative to seronegative individuals, this was only observed for $CD45RO^+CD27^+$ cells in the HIV-2 cohort. PD-1 expression on $CD4^+$ T cells was similarly linked to cell differentiation, with expression on naïve cells being significantly lower in HIV-1+ and HIV-2+ as compared to seronegative individuals; conversely, PD-1 expression on memory subsets was increased in both HIV cohorts, although only reaching statistical significance for HIV-1 (Supplemental Figure 1).

PD-1 expression on $CD4^+$ T cells was strongly associated with the degree of $CD4^+$ T-cell depletion and T-cell activation in both HIV cohorts (Table 1 and Supplemental Table 2). Additionally, significantly increased levels of PD-1 on $CD4^+$ and $CD8^+$ T cells were found in viremic as compared to both aviremic HIV-2+ and seronegative individuals (Supplemental Figure 2).

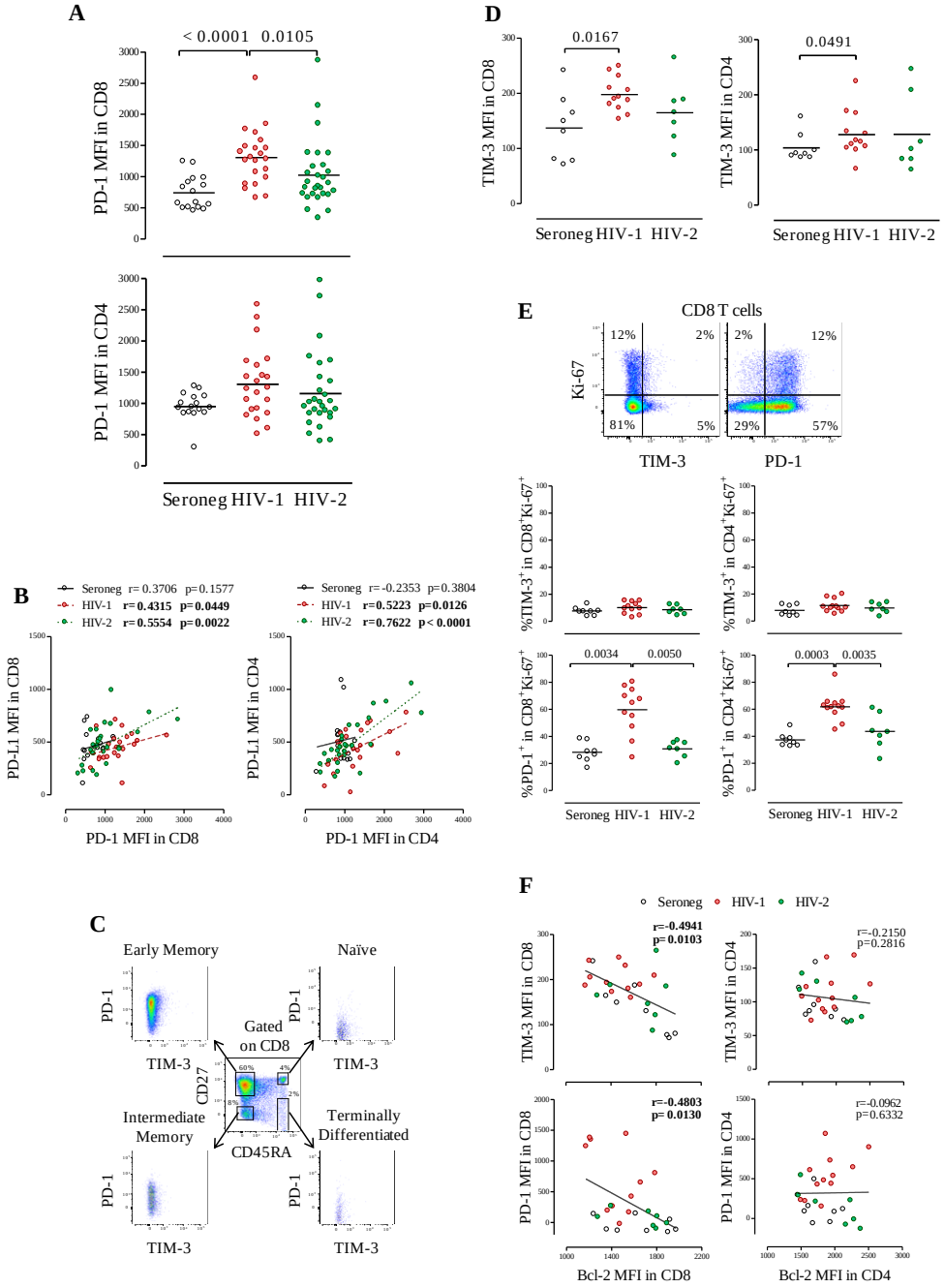

Figure 1. T-cell expression of PD-1, PD-L1 and TIM-3 in HIV-2 infection. (A) PD-1 Mean Fluorescence Intensity (MFI) assessed within total CD8$^+$ and CD4$^+$ T cells in seronegative, HIV-1 and HIV-2 cohorts. **(B)** Correlations between PD-1 and PD-L1 MFI within total CD8$^+$ and CD4$^+$ T cells in the 3 cohorts. **(C)** Illustrative dot-plots of PD-1 and TIM-3 expression within naïve and memory CD8$^+$ T-cell subsets, defined according to CD45RA and CD27, in an HIV-1+ patient with 650 CD4$^+$ T cells/µl and 21731 RNA copies/ml. **(D)** TIM-3 MFI within total CD8$^+$ and CD4$^+$ T cells in seronegative, HIV-1 and HIV-2 cohorts in early stage disease as described in Supplementary Digital Content, Table 3. **(E)** Dot-plots illustrate the analysis of TIM-3 and PD-1 in relation to Ki-67 within total CD8$^+$ T cells in the same patients as in **(C)**. The relative contribution of TIM-3$^+$ and PD-1$^+$ cells to the pool of cycling CD8$^+$ and CD4$^+$ T cells (Ki-67$^+$) are shown in the graphs. **(F)** Correlations between the expression of the pro-survival factor Bcl-2 and TIM-3 or PD-1 expression within CD8$^+$ and CD4$^+$ T cells in the 3 cohorts. Spearman correlation coefficients are shown. Each dot represents one individual. Bars represent mean.

Importantly, no statistically significant correlations were found between PD-1 expression levels on $CD8^+$ T cells and the aforementioned parameters in either HIV cohort, except for CD38 MFI within $CD8^+$ T cells (Table 1).

Table 1. *Correlations between PD-1 or PD-L1 expression (MFI) on $CD4^+$ and $CD8^+$ T cells and surrogate markers of HIV disease progression.*[a]

	PD-1 MFI					
	Seronegatives		HIV-1		HIV-2	
	CD4	CD8	CD4	CD8	CD4	CD8
% CD4 T cells	0.33;0.21	0.01;0.96	**-0.83;<0.0001**	-0.20;0.37	**-0.62;0.0004**	-0.20;0.30
$CD4^+$ T cells/µl	0.36;0.18	0.13;0.63	**-0.85;<0.0001**	-0.39;0.07	**-0.50;0.007**	-0.27;0.17
%HLA-DR^+ in CD4	0.19;0.49	0.32;0.23	**0.64;0.001**	0.19;0.39	**0.68;<0.0001**	0.30;0.11
CD38 MFI in CD8	-0.006;0.98	-0.10;0.72	**0.54;0.009**	**0.59;0.004**	**0.58;0.001**	**0.59;0.001**
Viremia (RNA cp/ml)[a]	NA	NA	**0.43;0.04**	0.41;0.05	NA	NA
	PD-L1 MFI					
	Seronegatives		HIV-1		HIV-2	
	CD4	CD8	CD4	CD8	CD4	CD8
% CD4 T cells	0.04;0.87	0.19;0.47	-0.42;0.05	**-0.43;0.04**	**-0.44;0.02**	**-0.43;0.02**
$CD4^+$ T cells/µl	0.01;0.98	-0.17;0.52	-0.31;0.16	-0.39;0.07	**-0.58;0.001**	**-0.46;0.01**
%HLA-DR^+in CD4	0.16;0.55	0.28;0.30	0.21;0.35	0.25;0.27	**0.54:0.003**	0.35;0.07
CD38 MFI in CD8	-0.15;0.57	-0.22;0.41	0.29;0.18	0.26;0.25	**0.58;0.001**	**0.40;0.03**
Viremia (RNA cp/ml)[b]	NA	NA	0.18;0.43	0.11;0.64	NA	NA

[a] Relationships were tested for significance with Spearman correlations: r;*p* values are shown. Results with *p* values <0.05 are highlighted in bold. NA, Not Applicable.
[b] For statistical analyses, half of the cut-off value was considered in the cases in which viremia was below the detection limits.

To investigate which of these factors most strongly influenced PD-1 expression on T cells, we conducted multiple linear regression (MLR) analyses using combined data from all cohorts. Colinearity analysis revealed associations between $CD4^+$ T-cell counts and T-cell activation levels (data not shown), in agreement with variable inter-dependency [21]. MLR analysis revealed a statistically significant association between PD-1 and HLA-DR expressions on $CD4^+$ T cells (β=0.444: *p*=0.030). Moreover, PD-1 expression levels within $CD45RO^+CD27^+$ $CD8^+$ T cells were significantly associated with CD38 expression on $CD8^+$ T cells (β=0.542: *p*=0.025) and nearly significantly associated

with viremia (β=0.286: *p*=0.055). Moreover, a canonical analysis further supported a role for immune activation in driving increased PD-1 expression on $CD4^+$ and $CD8^+$ T cells, as well as an additional contribution of viremia to PD-1 up-regulation on $CD8^+$ T cells (Supplemental Figure 2).

Overall, our results suggest that immune activation is the main factor contributing to the increased PD-1 levels during HIV infection. Additionally, they also indicate a role for viremia in PD-1 up-regulation on $CD8^+$ T cells, particularly on the $CD45RO^+CD27^+$ subpopulation.

PD-L1 expression on T cells

PD-L1 can also be up-regulated on T cells upon activation [30]. Strong direct correlations were observed in HIV-2+ individuals between PD-L1 expression on $CD4^+$ and $CD8^+$ T cells and both the degree of $CD4^+$ T-cell depletion and T-cell activation (Table 1 and Supplemental Table 2). Such correlations were largely absent in the HIV-1 cohort and non-existent in seronegative controls (Table 1), suggesting that PD-L1 expression on T cells increases specifically during HIV-2 disease progression. This was observed despite the lack of a significant increase in PD-L1 expression levels on $CD8^+$ and $CD4^+$ T cells in HIV-2 infection and of a similar differentiation-associated pattern of PD-L1 and PD-1 expression in all cohorts (Supplemental Figure 3). Moreover, the expression of PD-L1 and PD-1 expression within $CD4^+$ and $CD8^+$ T cells was apparently highly related (Figure 1B). Overall, our data revealed that HIV-2+ individuals showed strong correlations between PD-L1 and $CD4^+$ T-cell depletion and T-cell activation, suggesting that the strict control of PD-L1 expression may contribute to the slow HIV-2 disease progression.

TIM-3 expression on T cells

In order to investigate TIM-3 expression in HIV-2 infection and its relationship with PD-1/PD-L1, we studied additional age-matched cohorts of ART-naïve HIV-1+ and

HIV-2+ individuals with >300 $CD4^+$ T cells/µl, and seronegative controls (Supplemental Table 3 for clinical and epidemiological data).

The mechanisms regulating TIM-3 expression remain poorly understood [31-34]. Regarding the $CD8^+$ T-cell subset, the frequency of $TIM3^+PD1^+$ cells was very low (Figure 1C) and, in contrast to PD-1, TIM-3 expression seemed to be independent of T-cell differentiation in all cohorts (Supplemental Figure 4).

TIM-3 expression was higher in HIV-1+ patients as compared to both HIV-2+ and seronegative individuals on $CD8^+$ and $CD4^+$ T cells, although only reaching significance in comparison with the latter (Figure 1D).

Of note, the contribution of $TIM\text{-}3^+$ cells to cycling T cells was negligible in all cohorts in marked contrast to $PD\text{-}1^+$ cells (Figure 1E), which represented a significant proportion of cycling T cells, particularly in the HIV-1 cohort, further supporting the requirement of TCR stimulation for PD-1 up-regulation.

Conversely, both PD-1 and TIM-3 expression seemed to impact on $CD8^+$ T-cell survival, as previously described [35, 36], as indicated by the inverse correlation found with Bcl-2 expression levels within $CD8^+$ T cells (Figure 1F). No such correlations were found on $CD4^+$ T cells (Figure 1F).

We also confirmed that the above reported imbalances in the expression of inhibitory molecules were unrelated to regulatory $CD4^+$ T cells (Treg), defined by FoxP3 expression (Supplemental Figure 5) that have been reported to express high levels of PD-1 and TIM-3 [37, 38].

Overall, our data showed that over-representation of a $TIM\text{-}3^+$ population in T cells, particularly within the $CD8^+$ subset, was much more marked in HIV-1 than in HIV-2 infection. Moreover, TIM-3 expression was apparently independent of T-cell differentiation and disassociated from cell-cycling.

Discussion

We report here, for the first time, that HIV-2 infection was associated with increased PD-1/PDL-1 expression on both $CD4^+$ and $CD8^+$ T cells, in direct correlation with the degree of $CD4^+$ T-cell depletion and T-cell activation. PD-1 up-regulation occurred despite the low to undetectable viremia found in the majority of untreated HIV-2+ patients. We also found strong correlations between the expression of its main ligand, PD-L1, within both $CD4^+$ and $CD8^+$ T cells, and the degree of $CD4^+$ T-cell depletion and immune activation that were exclusive to the HIV-2 cohort, in agreement with our previous data on pDC [23]. The expression of these inhibitory markers seemed to be mainly driven by immune activation, the major determinant of the rate of $CD4^+$ T-cell loss and AIDS progression, both in HIV-1 and HIV-2 infections [5, 8, 39, 40]. Our data suggest that PD-1/PD-L1 up-regulation may reflect an attempt to limit immunopathology, hence contributing to a more “benign” course of HIV infection.

We showed that HIV-2+ individuals did not feature a significant up-regulation of TIM-3, in contrast to the HIV-1 cohort which exhibited an over-expression of TIM-3 within $CD4^+$ and $CD8^+$ T cells, as previously reported [15, 16], even in early stage disease. TIM-3 expression was dissociated from PD-1 expression, and markers of cell differentiation and cell-cycling, but inversely correlated with the levels of the anti-apoptotic molecule Bcl-2 on $CD8^+$ T cells. Thus, PD-1 and TIM-3 may be differentially regulated, resulting in their expression by functionally distinct T-cell populations. Overall, we found no significant changes in TIM-3 expression in HIV-2+ individuals, in contrast to its up-regulation throughout HIV-1 infection, suggesting a role of TIM-3 in the protected HIV-2 disease course.

In conclusion, we showed that, during the relatively “benign” course of HIV-2 infection, there was a progressive increase in the expression of PD-1 and PD-LI within $CD4^+$ and $CD8^+$ T cells but not of TIM-3. Our data suggest that the PD-1/PD-L1 pathway may counteract the hyper-immune activation associated with AIDS progression with implications for the design of ART-complementary immune-based strategies.

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Supplemental Data

Supplemental Table 1. *Cohort clinical and epidemiological data.*[a]

	Seronegatives	HIV-1	HIV-2
Numbers (male/female)	16 (6/10)	22 (17/5)	28 (9/19)
Age (years)	44 ± 2 (27-57)	40 ± 2 (23-61)	49 ± 3 (19-78)
Ethnicity (Caucasian/other)	15/1	13/7	14/14
% $CD3^+$ cells	62.6 ± 1.9 (49-76.3)	67.4 ± 2.1 (48.6-82.8)	63.8 ± 2.0 (46.3-87.4)
$CD3^+$ cells/µl	1286 ± 93 (695-1929)	1621 ± 171 (538-3970)	1399 ± 112 (346-2930)
% $CD4^+$ T cells	52.4 ± 2.5 (5.36-67.0)	24.6 ± 3.9 (1.7-70.1)***	31.6 ± 3.1 (6.8-62)***
$CD4^+$ T cells/µl	906 ± 59 (518-1312)	569 ± 105 (18-1848)*	659 ± 81 (52-1511)*
% HLA-DR^+ in CD4	4.1 ± 0.4 (1.9-7.6)	17.1 ± 3.0 (1.7-54.5)***	11.4 ± 1.7 (1.8-36.3)**
% $CD8^+$ T cells	24.0 ± 1.8 (11.7-35.2)	46.7 ± 2.8 (12.4-64.7)***	38.1 ± 2.3 (21-65.7)***#
$CD8^+$ T cells/µl	503 ± 51 (212-802)	1107 ± 120 (138-2514)***	816 ± 75 (221-1817)**#
CD38 MFI in CD8	1237 ± 106 (838-2539)	5128 ± 848 (706-13553)***	2854 ± 537 (643-13659)* #
Viremia (RNA cp/ml)	NA	778459 ± 334956 (71-4.5x10^6)[b]	11266 ± 3,633 (1189-3.6x10^4)[c]

[a]Data are Mean±SEM with limits in brackets, unless indicated otherwise. NA, Not Applicable. Data referring to the early stage cohort are not included in this Table (see Supplemental Table 3). Statistical differences between a given HIV cohort and the seronegative controls: *, $p < 0.05$; **, $p < 0.01$; ***, $p<0.001$. Statistical differences between HIV-1+ and HIV-2+ individuals: #, $p < 0.05$.
[b] HIV-1 viremia was < 40 RNA copies/ml (cutoff) in 3 of the 22 subjects studied.
[c] HIV-2 viremia was < 200 RNA copies/ml (cutoff) in 21 of the 28 subjects studied.

Supplemental Table 2. *Correlations between PD-1 or PD-L1 MFI on CD4$^+$ and CD8$^+$ T cells and additional surrogate markers of HIV disease progression.*[a]

	PD-1 MFI					
	Seronegatives		HIV-1		HIV-2	
	CD4	CD8	CD4	CD8	CD4	CD8
HLA-DR MFI in CD4	0.36;0.17	0.40;0.13	**0.70;0.0003**	0.36;0.10	**0.67;<0.0001**	**0.45;0.02**
%HLA-DR$^+$ in CD8	0.16;0.55	0.17;0.52	**0.47;0.02**	**0.43;0.04**	**0.63;0.0004**	**0.37;0.05**
HLA-DR MFI in CD8	0.23;0.39	0.21;0.43	0.35;0.12	**0.58;0.005**	**0.60;0.0008**	**0.47;0.01**
%CD38$^+$ in CD8	-0.11;0.68	-0.11;0.67	**0.55;0.009**	**0.50;0.02**	**0.46;0.01**	**0.44;0.02**
β2-microglobulin (mg/l)[b]	ND	ND	0.38; 0.18	0.10;0.72	**0.57;0.0075**	0.35;0.12
	PD-L1 MFI					
	Seronegatives		HIV-1		HIV-2	
	CD4	CD8	CD4	CD8	CD4	CD8
HLA-DR MFI in CD4	0.17;0.53	0.41;0.11	0.28;0.21	0.28;0.20	**0.53;0.004**	0.32;0.09
%HLA-DR$^+$ in CD8	0.16;0.54	-0.01;0.97	**0.56;0.01**	**0.52;0.01**	**0.51;0.005**	**0.39;0.04**
HLA-DR MFI in CD8	0.11;0.68	0.01;0.98	0.41;0.06	**0.44;0.04**	**0.49;0.008**	0.34;0.07
%CD38$^+$ in CD8	-0.31;0.25	-0.37;0.16	0.28;0.20	0.20;0.37	**0.53;0.004**	0.33;0.08
β2-microglobulin (mg/l)[b]	ND	ND	0.27;0.35	-0.01;0.98	**0.66;0.001**	**0.55;0.01**

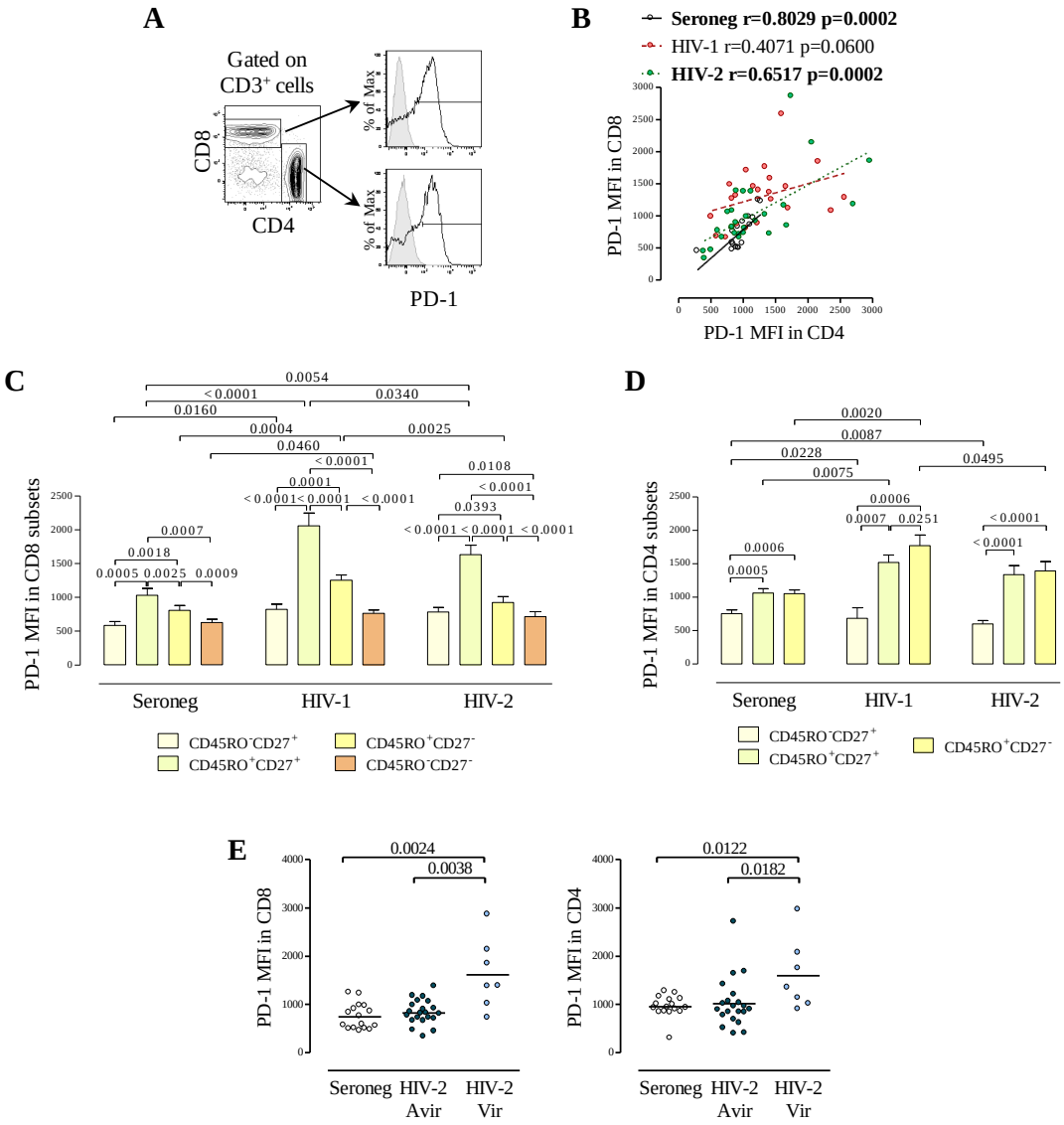
[a] Relationships were tested for significance with Spearman correlations: r;*p* values are shown. Results with *p* values <0.05 are highlighted in bold. ND: Not Determined.

[b] β2-microglobulin serum levels were assessed for 21 HIV-2 (3.0±0.4 mg/l) and 14 HIV-1 (3.0±0.4 mg/l) infected individuals, with no statistically significant differences having been observed between cohorts.

Supplemental Table 3. *Clinical and epidemiological data of the early stage cohorts.*[a]

	Seronegatives	HIV-1	HIV-2
Numbers (male/female)	8 (3/5)	12 (8/4)	7 (1/6)
Age (years)	42 ± 5 (26-57)	41 ± 3 (25-63)	50 ± 5 (33-66)
Ethnicity (Caucasian/other)	8/0	9/3	5/2
% $CD3^+$ cells	67.3 ± 3.8 (51.8-88.1)	69.7 ± 2.3 (56.6-86.7)	72.3 ± 2.1 (63.5-78.6)
$CD3^+$ cells/µl	1725 ± 307 (927-3135)	1730 ± 130 (1102-2756)	1547 ± 197 (1126-2517)
% $CD4^+$ T cells	36.3 ± 3.6 (26.2-51.9)	26.9 ± 2.6 (19.4-50.1)*	31.6 ± 3.1 (19.5-45.7)
$CD4^+$ T cells/µl	905 ± 118 (512-1385)	662 ± 74 (430-1361)	697 ± 143 (335-1464)
% HLA-DR^+ in CD4	5.8 ± 0.9 (3.8-10.3)	6.7 ± 1.2 (1.5-13.7)	5.9 ± 1.4 (3.1-11.6)
% $CD8^+$ T cells	30.4 ± 5.4 (13.2-61.1)	42.1 ± 2.9 (26.3-67)*	40.4 ± 2.9 (28.1-47.7)
$CD8^+$ T cells/µl	808 ± 289 (302-2175)	1054 ± 104 (493-1887)	834 ± 76 (526-1130)
CD38 MFI in CD8	2447 ± 298 (1394-3993)	4895 ± 626 (1865-9787)**	3098 ± 220 (2454-3665)
Viremia (RNA cp/ml)	NA	25,949 ± 8405 (295-73,110)	NA[b]

[a] Data are Mean±SEM with limits in brackets, unless indicated otherwise. NA, Not Applicable. Statistical differences between a given HIV cohort and the seronegative controls: *, $p < 0.05$; **, $p < 0.01$.
[b] HIV-2 viremia was < 200 RNA copies/ml (cutoff) in all of the subjects studied.



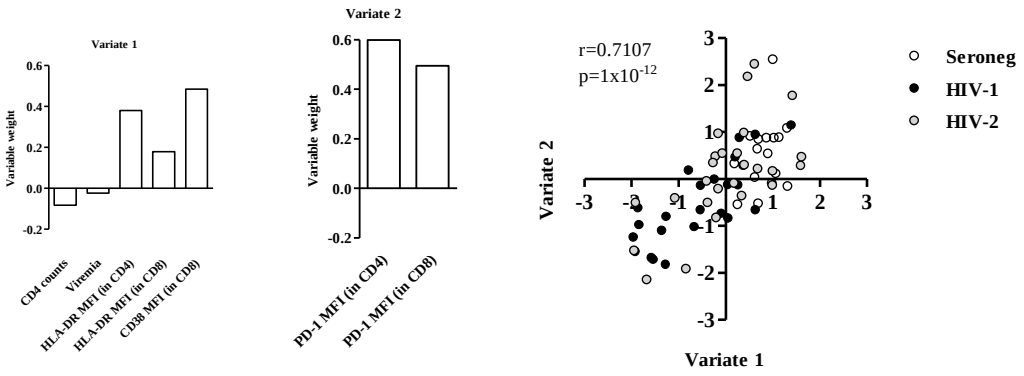
Supplemental Figure 1. PD-1 expression according to cell-differentiation and viremia in HIV-2+ individuals. (A) Gating strategy used to assess PD-1 (clone: MIH-4) expression by flow cytometry: cells were first gated according to CD8 or CD4 expression (within a $CD3^+$ gate) and PD-1 expression was then assessed using as reference a "fluorescence minus one" control (grey histogram). **(B)** Similarly to seronegative controls, HIV-2+ individuals exhibited a significant correlation between PD-1 expression on $CD8^+$ and on $CD4^+$ T-cell subsets. PD-1 expression was assessed within naïve and memory $CD8^+$ **(C)** and $CD4^+$ **(D)** T-cell subsets defined according to CD45RO and CD27 expression. Bars represent mean±SEM and statistically significant differences are shown. **(E)** PD-1 expression in HIV-2+ individuals stratified according to levels of circulating virus: undetectable (aviremic, avir) and viremic (vir). Each dot represents one individual.

Supplemental Figure 2. *Canonical correlation analysis.*

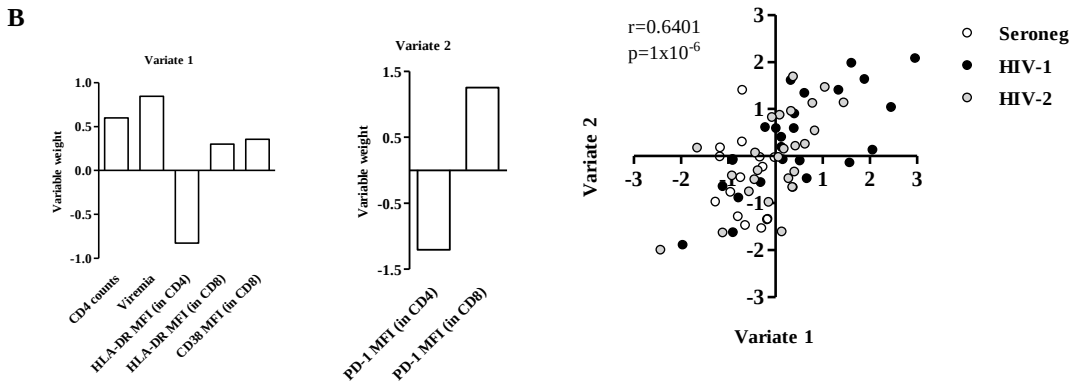
As none of the MLR models fully explained the variability in PD-1 expression seen among the different T-cell subsets (r^2<0.6 for all models), a canonical correlation analysis was performed in order to clarify the determinants of PD-1 expression within T cells.

This technique looks for correlations between linear combinations of a set of predictive variables and of outcome variables, commonly denominated variates or canonical variables. In our analysis, the predictive variate (variate 1) included $CD4^+$ T-cell counts, viral load and T-cell activation (HLA-DR expression on $CD4^+$ T cells and HLA-DR and CD38 expression on $CD8^+$ T cells, measured as MFI), and the outcome variate (variate 2) PD-1 expression (MFI) on $CD4^+$ and $CD8^+$ T cells in all the individuals studied. The two most significant correlations found are shown.

A

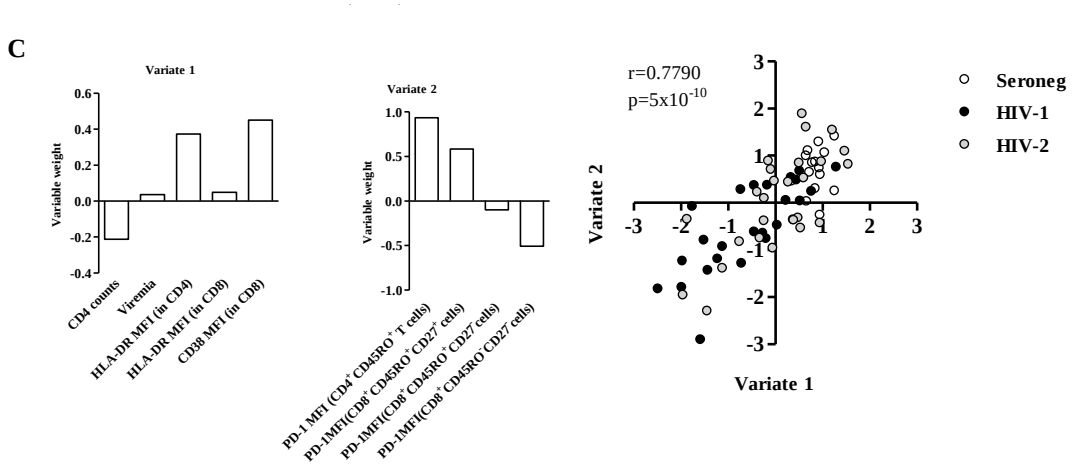


The first **(A)** shows a strong association between a predictive variate dominated by T-cell activation parameters and a outcome variate with high values for PD-1 expression on both total $CD4^+$ and $CD8^+$ T cells, which implicates immune activation as the major determinant of PD-1 expression on both T-cell subsets.

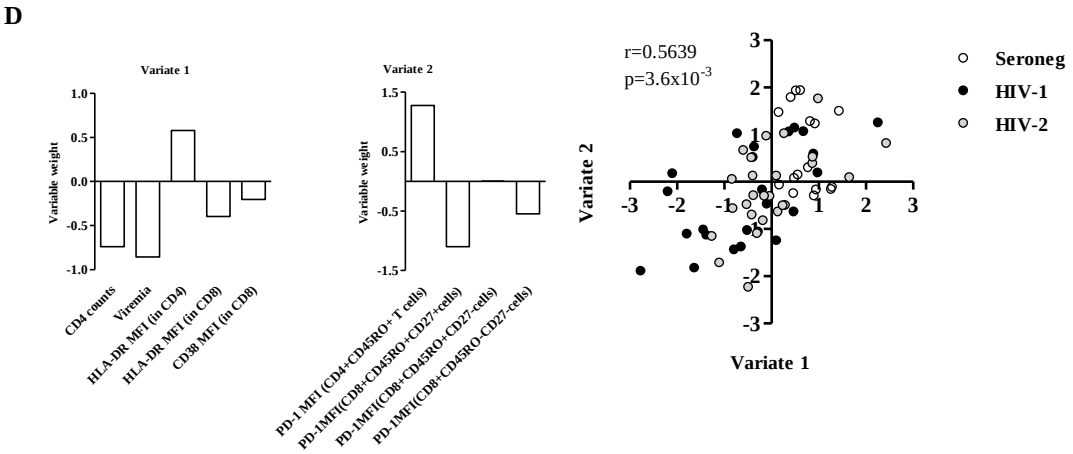


The second **(B)** represents an association between low HLA-DR expression on $CD4^+$ T cells and high viremia with low PD-1 expression on $CD4^+$ T cells and high PD-1 expression on $CD8^+$ T cells, thus reinforcing the role of immune activation in driving PD-1 over-expression but also indicating a contribution of viremia in the up-regulation of PD-1 on $CD8^+$ T cells.

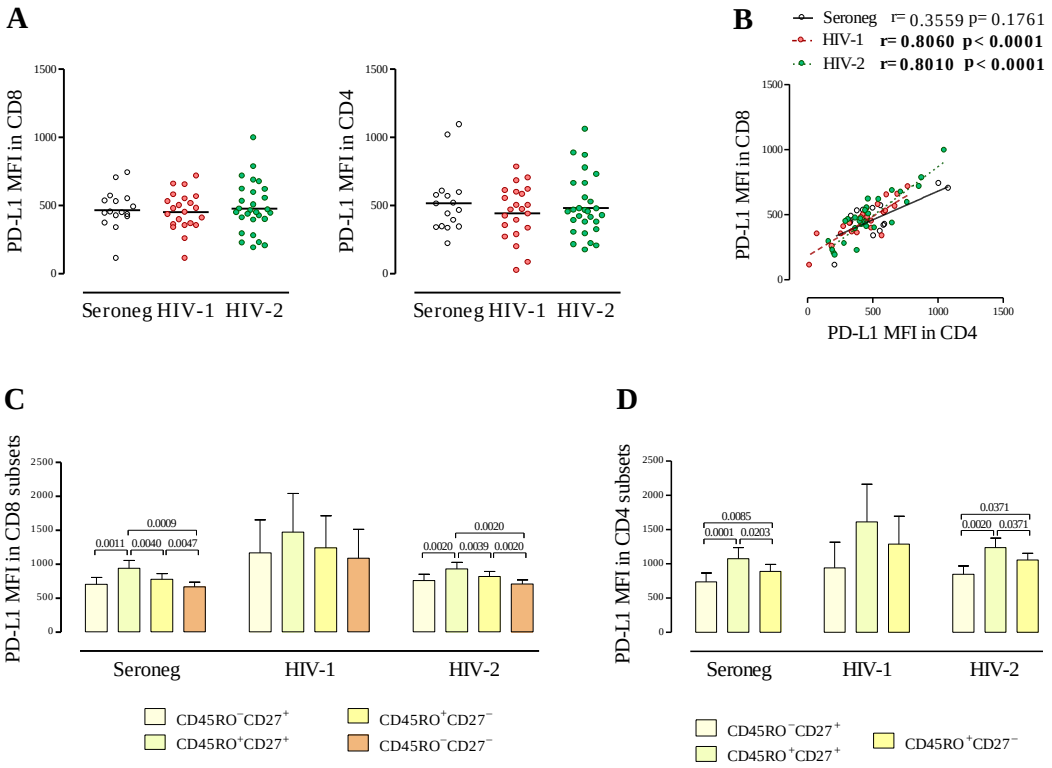
Colinearity analysis failed to reveal any significant association between PD-1 expression on the different T-cell subpopulations, which suggests that PD-1 expression is regulated in a subset-independent manner. To address the regulation of PD-1 expression within the different T-cell subpopulations, we performed an additional canonical correlation analysis in which disease progression markers (variate 1) were correlated to PD-1 expression on memory-effector $CD4^+$ and $CD8^+$ T-cell subsets (variate 2 – among memory $CD4^+$ T cells, $CD45RO^+$ cells were considered as a whole as no difference in PD-1 expression was observed between $CD27^+$ and $CD27^-$ counterparts). The two most significant correlations found are shown.



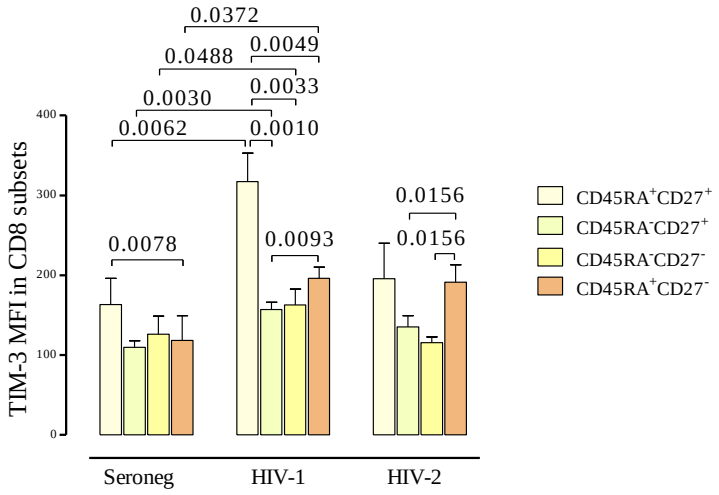
The first **(C)** demonstrates a relationship between a predictive variate combining mostly T-cell activation parameters and a outcome variate combining PD-1 expression within $CD4^+CD45RO^+$ and $CD8^+CD45RO^+CD27^+$ T cells.



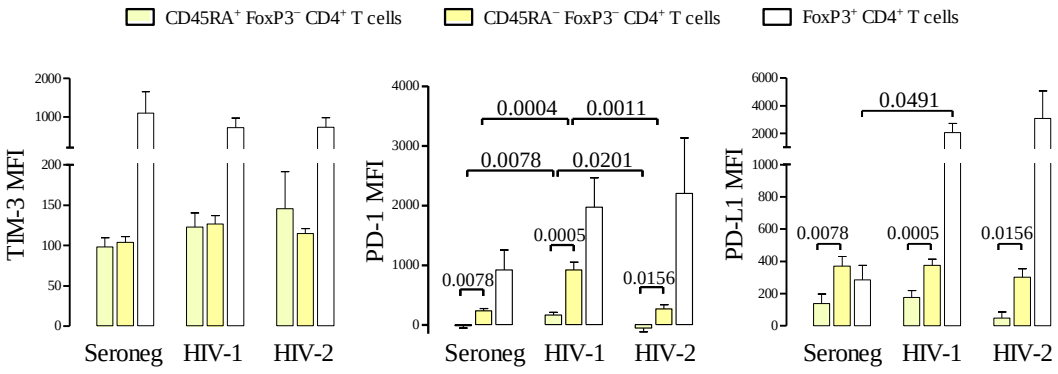
The second **(D)** represents direct associations between $CD4^+$ T-cell counts, viremia and PD-1 expression on $CD8^+CD45RO^+CD27^+$ T cells and $CD4^+$ T-cell activation (HLA-DR expression) and PD-1 expression on memory $CD4^+$ T cells. Overall, this analysis supports a major role for T-cell activation in PD-1 up-regulation on both $CD4^+$ and $CD8^+$ memory T cells and a contribution of $CD4^+$ T-cell depletion and viremia to PD-1 over-expression on $CD8^+CD45RO^+CD27^+$ T cells.



Supplemental Figure 3. PD-L1 expression on T cells. (**A**) PD-L1 MFI (clone MIH-1) within total $CD8^+$ and $CD4^+$ T cells in seronegative, HIV-1 and HIV-2 cohorts. Bars represent mean and each dot represents one individual. (**B**) Direct correlations were found in both infected cohorts between PD-L1 expression levels within $CD4^+$ and $CD8^+$ T cells. PD-L1 expression was further addressed on naïve and memory $CD8^+$ (**C**) and $CD4^+$ **(D)** T-cell subsets in a group consisting of 14 seronegative, 5 HIV-1+ and 10 HIV-2+ individuals. Naïve and memory T-cell subsets were defined according to CD45RO and CD27 expression and PD-L1 Mean Fluorescence Intensity (MFI) was then assessed within each cell population. Bars represent mean±SEM.



Supplemental Figure 4. TIM-3 expression on CD8$^+$ T-cell subsets. Graph shows TIM-3 MFI (clone: 344823) within each CD8$^+$ T-cell sub-population in all cohorts. Bars represent mean±SEM.



Supplemental Figure 5. TIM-3, PD-1 and PD-L1 expression on naïve Foxp3$^-$, memory Foxp3$^-$ and regulatory (FoxP3$^+$) CD4$^+$ T cells. TIM-3 (left), along with PD-1 (middle) and PD-L1 (right) MFI was assessed within CD45RA$^+$FoxP3$^-$ and CD45RA$^-$FoxP3$^-$ populations and FoxP3$^+$ CD4$^+$ T cells in seronegative, HIV-1+ and HIV-2+ individuals. Bars represent mean±SEM. The results obtained with naïve and memory FoxP3$^-$ subsets were broadly similar to those observed within total CD4$^+$ T cells. Regarding the Treg population, both HIV-2 and HIV-1 cohorts featured significantly higher levels of PD-1 expression than the seronegative individuals, but TIM-3 levels were similar in all cohorts. Also, a preliminary analysis revealed a strong correlation between PD-L1 MFI on CD4$^+$ T cells and the frequency of FoxP3-expressing CD4$^+$ T cells in the HIV-2 (p=0.007, r=0.881, n=8) but not in the HIV-1 cohort (p=0.808, r=-0.143, n=6).

3.3. Preserved CD4$^+$ T-cell telomere length during long-lasting HIV-2 infection

Submitted for publication:

Rita Tendeiro, Adriana S. Albuquerque, Russell B. Foxall, Rita Cavaleiro, Rui S. Soares, António P. Baptista, Maria V. D. Soares, Perpétua Gomes, Ana E. Sousa. **Preserved CD4 T-cell telomere length during long-lasting HIV-2 infection**.

This work was supported by grant (PTDC/SAU-MIC/109786/2009) from "Fundação para a Ciência e a Tecnologia" (FCT) and by "Programa Operacional Ciência e Inovação 2010" (POCI2010), as well as by Fundação Calouste Gulbenkian to AES. RT, ASA, RBF, RC, and RSS received scholarships from FCT.

Abstract

HIV-2 features a much slower course than HIV-1 infection, often asymptomatic for over 20 years without antiretroviral therapy (ART). Nevertheless, $CD4^+$ T cells progressively decline, in direct correlation with immune activation and cell-cycling. We reported, for the first time, preserved telomere length within naïve and memory $CD4^+$ T-cell subsets in prolonged HIV-2 infection despite the increased $CD4^+$ T-cell turnover.

Research letter

HIV infection is characterized by progressive $CD4^+$ T-cell depletion, associated with persistently high levels of T-cell turnover [1]. HIV-2, as compared to HIV-1 infection, is associated with a much slower $CD4^+$ T-cell decline and, consequently, prolonged disease length [2]. Nevertheless, when matched for the degree of $CD4^+$ T-cell depletion, HIV-2+ and HIV-1+ individuals show similar levels of T-cell activation and cycling [3, 4]. In HIV-1+ patients there is evidence of extensive $CD8^+$ T-cell replicative history with telomere shortening [5-7], though data on $CD4^+$ T cells remain controversial [8-10].

We investigated, for the first time, whether increased $CD4^+$ T-cell turnover and prolonged chronic inflammation in HIV-2+ individuals leads to significant telomere attrition. $CD4^+$ T-cell telomere length was estimated by three-colour flow-FISH (fluorescence *in situ* hybridization) in thawed peripheral blood mononuclear cells (PBMC), as previously described [11]. Blood samples were obtained after written consent and study was approval by the Ethical Board of the Faculty of Medicine of the University of Lisbon.

HIV-2 and HIV-1 cohorts featured comparable levels of $CD4^+$ T-cell depletion (HIV-2: 647±84 cells/µl, 28.3±2.8%; HIV-1: 530±94 cells/µl, 21.3±2.5%; and seronegatives: 950±71 cells/µl; 42.7±1.9%. $p<0.01$ for both HIV cohorts as compared to seronegatives); and hyper-immune activation (HIV-2: 4.4±0.9%, HIV-1: 7.4±1.1%, and seronegatives: 1.6±0.2% for frequency of HLA-DR^+CD38^+ in $CD4^+$ T cells; HIV-2: 16.1±2.3%, HIV-1: 19.5±2.8%, and seronegatives: 5.4±1.1% for frequency of HLA-DR^+CD38^+ in $CD8^+$ T cells. $p<0.01$ for both HIV cohorts as compared to seronegatives), but viremia was strikingly lower in HIV-2+ patients, who included 11 antiretroviral therapy (ART)-naïve individuals with undetectable viremia (HIV-2: $5.8x10^3$±$1.5x10^3$ and HIV-1: $6.6x10^5$±$6.5x10^5$ RNA copies/ml).

We found no significant differences in telomere length of circulating $CD4^+$ T cells from HIV-2+ and seronegative individuals (Figure 1A). As expected [12], and validating the assay, memory $CD4^+$ T cells showed significantly shorter telomeres than

naïve in both cohorts (Figure 1A; p<0.0001). Of note, telomere length of naïve and memory CD4$^+$ T cells did not differ between HIV-2 and seronegative cohorts. Thus, despite the prolonged length of HIV-2 infection [2] and the evidence of increased immune activation, no major CD4$^+$ T-cell telomere attrition was found.

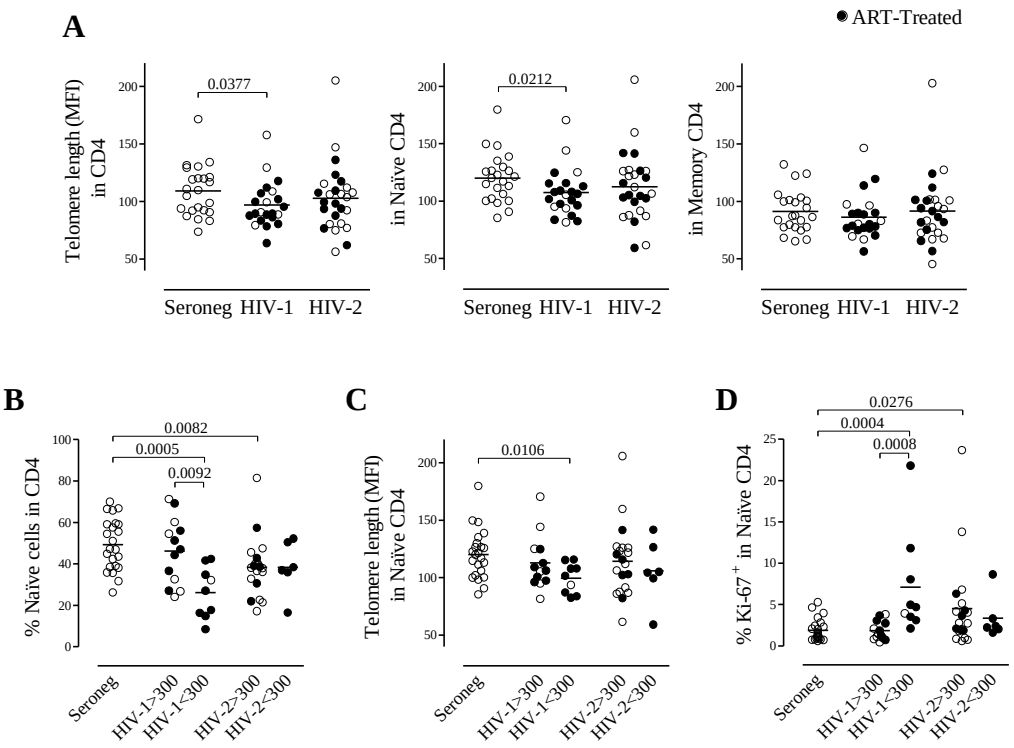


Figure 1. CD4$^+$ T-cell telomere length during HIV-2 infection. (A) Telomere length was assessed, using a Flow-FISH assay, in terms of Median Fluorescence Intensity (MFI) of whole CD4$^+$ T cells (left graph), naïve (CD45RO$^-$, central graph) and memory (CD45RO$^+$, right graph) CD4$^+$ T cells in HIV-2+ and HIV-1+, as well as seronegative (Seroneg) individuals. The HIV cohorts were further stratified, according to the degree of CD4$^+$ T-cell depletion, into subgroups of patients with > or < 300 CD4$^+$ T cells/µl and analysed in terms of: **(B)** Frequency of naïve cells within total CD4$^+$ T cells; **(C)** Telomere length (MFI) of naïve CD4$^+$ T cells; **(D)** Proportion of cycling cells within naïve CD4$^+$ T cells, assessed by the expression of Ki-67. Each symbol represents one individual and bars represent mean; closed symbols refer to ART-treated patients. Data were compared using Mann-Whitney tests. *P*-values below 0.05 were considered significant and are indicated for all statistical differences observed.

Conversely, HIV-1+ individuals featured significantly decreased CD4$^+$ T-cell telomere length, as compared to seronegative controls (Figure 1A), in close association with the frequency of circulating CD4$^+$ T cells (r=0.4320; p=0.0447).

HIV-2+ individuals tended to be older (HIV-2: 49±2years; HIV-1: 44±3years; seronegatives: 43±3years; not statistically different), a factor known to be associated

with shorter telomeres [13], stressing the relevance of our finding of preserved telomere length in the HIV-2 cohort. Additionally, and in agreement with epidemiological trends [14], our HIV-2 cohort was enriched in non-Caucasians (12/26; HIV-1: 1/22; seronegatives: 1/23) and females (13/26; HIV-1: 5/22; seronegatives: 18/23). As ethnicity and gender have also been described to impact on telomere length, although with conflicting results [15-17], linear regression and one-way analysis of variance were performed, which indicated a lack of significant effects of age, ethnicity and/or gender on the results obtained for telomere length in our HIV-2 cohort ($p>0.05$).

Of note, the overall reduction of $CD4^+$ T-cell telomere length in HIV-1 infection seemed to be related to preferential telomere erosion in the naïve subset (Figure 1A), with no differences being observed regarding memory $CD4^+$ T cells.

HIV infection is associated with progressive naïve $CD4^+$ T-cell loss. The ability to sustain this subset is thought to affect the size and diversity of the overall $CD4^+$ T-cell pool, possibly contributing to slower HIV disease progression. Both HIV cohorts exhibited a decline of naïve cells within total $CD4^+$ T cells, which was particularly marked in advanced HIV-1 infection (<300 $CD4^+$ T cells/µl) (Figure 1B). Accordingly, in HIV-1+ individuals, the proportion of naïve cells was inversely associated with $CD4^+$ T-cell depletion ($CD4^+$ T-cell counts: $r=0.7134$, $p=0.0002$; % of $CD4^+$ T cells: $r=0.8080$, $p<0.0001$) and T-cell activation (% of HLA-DR^+CD38^+ in $CD4^+$ T cells: $r=-0.8512$, $p<0.0001$; and in $CD8^+$ T cells: $r=-0.4704$, $p=0.0272$), which did not occur in the HIV-2 cohort ($p>0.05$ for all the aforementioned parameters).

Notably, HIV-1+ individuals in advanced disease stage (<300 $CD4^+$ T cells/µl) featured the lowest naïve $CD4^+$ T-cell telomere length (Figure 1C). All but one of these patients were on long-term ART (44±8months, n=8), with undetectable viremia despite poor immunological recovery, and have been included in a previously described ART-discordant cohort [3, 18]. Our findings are in agreement with the reported impairment of thymic function that has been linked with discordant responses to ART in HIV-1 infection [19, 20]. On the other hand, our data suggest that, in advanced HIV-2 infection, there is a better maintenance of naïve $CD4^+$ T-cell

telomere length (Figure 1C). The sustainability of the naïve compartment may be related to a relatively preserved thymic activity during HIV-2 infection, as we reported [21]. Of note, the HIV-2 cohort also included 12 ART-treated patients, that, as previously described [22], showed significant $CD4^+$ T-cell depletion (360±69 cells/µl; 6/12 patients with <300 $CD4^+$ T cells/µl) despite low viremia (undetectable in 9/12 patients), in agreement with the poor immunological recovery upon ART usually observed in HIV-2 infection [2, 22-24].

Naïve $CD4^+$ T-cell homeostasis also relies on peripheral homeostatic proliferation [25]. The frequency of cycling cells within the naïve $CD4^+$ T-cell pool, estimated by the cell-cycle marker Ki-67 as previously described [25], was markedly increased in both HIV cohorts, in relation to seronegatives (Figure 1D), although significantly lower than those observed within memory $CD4^+$ T cells (HIV-2: 18.1±2.1%; HIV-1: 18.2±2.8%; seronegatives: 8.9±0.7%; $p<0.0001$ for the comparison between frequency of cycling naïve and memory $CD4^+$ T cells in all cohorts; $p<0.001$ for the comparison of proportion of cycling memory cells between both HIV and seronegative cohorts). The proportion of cycling cells within naïve $CD4^+$ T cells was more noticeably elevated in patients with advanced HIV-1 infection (<300 $CD4^+$ T cells/µl), reaching statistical significance both in comparison with seronegatives and corresponding early stage disease group (Figure 1D).

Thus, in HIV-1+ individuals with advanced disease, maintenance of naïve $CD4^+$ T cells seemed to critically depend upon homeostatic peripheral expansion. In contrast, no significant increase in the frequency of cycling cells was observed in advanced HIV-2 disease with similar levels of immune activation [4].

Our data further support a contribution of thymopoiesis throughout the course of HIV-2 infection that may help limit the degree of telomere attrition in the naïve $CD4^+$ T-cell compartment. The latter could also be related to maintained telomerase activity in HIV-2 infection. This possibility warrants further investigation in HIV-2+ patients, given the evidence indicating their preserved responsiveness to IL-7 [26], an important telomerase inducer [27].

Overall, this study revealed that prolonged HIV-2 infection is associated with preserved CD4+ T-cell telomere length, despite increased immune activation and lymphocyte turnover.

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3.4. Preserved naïve CD4$^+$ T-cell ability to proliferate upon IL-7 stimulation distinguishes HIV-2 from HIV-1 infection

Manuscript in preparation:

Rita Tendeiro, Russell B. Foxall, Rui S. Soares, Emília Valadas, Perpétua Gomes, Rui M. M. Victorino, Ana E. Sousa. **Preserved naïve CD4$^+$ T-cell ability to proliferate upon IL-7 stimulation distinguishes HIV-2 from HIV-1 infection**.

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Abstract

Strategies to preserve and/or recover naïve $CD4^+$ T cells are considered critical in helping achieve immune-competence in HIV infection. The maintenance of the naïve compartment after thymic export largely relies on IL-7 induced cell proliferation, which preferentially expands the $CD31^+$ subset that includes recent thymic emigrants (RTE). We investigated here how IL-7 responsiveness disturbances impact upon naïve $CD4^+$ T-cell homeostasis in HIV/AIDS.

Our results revealed that distinct mechanisms contributed to naïve $CD4^+$ T-cell maintenance in HIV-2 and HIV-1 infections. HIV-2+ patients, typically characterized by a much slower $CD4^+$ T-cell decline and reduced viremia, featured a significant over-representation of the $CD31^+$ subset within cycling naïve $CD4^+$ T cells, both in relation to age-matched HIV-1+ and seronegative individuals. Furthermore, when naïve $CD4^+$ T cells from HIV-2+ patients were cultured in the presence of IL-7, their ability to proliferate was well-preserved. This was not the case for HIV-1+ patients, whose naïve $CD4^+$ T cells, particularly the $CD31^+$ subset, featured impaired IL-7-mediated proliferative responses. These differences were neither related to CD127 expression nor circulating IL-7 levels.

Overall, we provide evidence supporting a sustained ability of naïve $CD4^+$ T cells to proliferate in response to IL-7 in HIV-2+ individuals. The identification of the mechanisms underlying the differences in IL-7 responsiveness in the two HIV infections may help design novel ART-complementary therapeutic approaches.

Introduction

HIV infection is characterized by progressive CD4$^+$ T-cell depletion [1], eventually leading to immunological failure and, ultimately, death. This depletion involves not only memory CD4$^+$ T cells, the main targets of HIV infection, but also naïve cells, including recent thymic emigrants (RTE) [2].

The maintenance of the peripheral CD4$^+$ T-cell pool is known to depend on a continuous replenishment from the thymus that is constrained by the age-related decline in thymopoiesis [3]. Moreover, increasing evidence points to an important role of naïve T-cell proliferation in the establishment and preservation of the peripheral naïve CD4$^+$ T-cell compartment [4, 5].

Studies have shown that, during HIV-1 infection, naïve CD4$^+$ T cells are preferentially depleted in lymphoid tissues [6-8], indicating a disturbance in naïve T-cell homeostasis. Hence, elucidation of the processes underlying naïve CD4$^+$ T-cell homeostasis during HIV disease is required, in order to better understand HIV/AIDS pathogenesis, and for the development of new strategies to enhance the quality of immune reconstitution following anti-retroviral therapy (ART).

When leaving the thymus, mature naïve CD4$^+$ T cells, also known as RTE, feature high levels of T-cell receptor excision circles (TREC) and express the surface marker platelet endothelial cell adhesion molecule-1 (PECAM-1, or CD31) [9]. Maintenance of naïve CD4$^+$ T cells in the periphery relies on a significant degree of homeostatic proliferation, which is thought to involve both self-peptide/MHC-TCR interactions and IL-7, a member of the common γ chain (γc) cytokine family [10]. We have recently reported that IL-7 preferentially expands the CD31$^+$ subset of naïve CD4$^+$ T cells in healthy adults [11]. Moreover, although the loss of CD31 expression is thought to be linked to cell cycling, we have shown that cells proliferating in response to IL-7 preserve CD31 expression [11]. Additionally, IL-7 was observed to maintain, or even increase, the levels of CD31 expression on non-proliferating cells [11]. Thus, it is likely that IL-7 plays a crucial role in the maintenance of CD31$^+$ naïve CD4$^+$ T cells during adulthood, thereby helping to sustain TCR diversity within CD4$^+$ T cells. Of note,

HIV-1 infection has been associated with functional defects in naïve T cells, namely decreased IL-7Rα chain expression [12, 13] and impaired responsiveness to IL-7 stimulation [14-16] despite the increased serum IL-7 levels [17].

HIV-2 infection, a naturally-occurring form of attenuated HIV disease, is characterized by a very slow rate of $CD4^+$ T-cell depletion [18], undetectable to low levels of circulating virus [19], and a generally more "benign" disease course [20].

Here we hypothesize that the slower HIV-2 disease progression is related to a better ability of HIV-2+ individuals to replenish their naïve $CD4^+$ T-cell pool from early in the infection. Previous data from our group indicated that HIV-2+ individuals may feature a better preserved response to γc cytokines [21-23]. Specifically, we have shown that, in comparison with their HIV-1+ counterparts, HIV-2+ individuals feature a delayed increase in IL-7 levels during disease progression, and a better preservation of the IL-7Rα chain expression, particularly in the naïve $CD4^+$ T-cell pool [21], suggesting a maximization of available resources in the context of this disease.

We found that HIV-2+ patients presented a significant over-representation of $CD31^+$ cells within the *ex vivo* pool of cycling naïve $CD4^+$ T cells relative to HIV-1+ and seronegative individuals. Furthermore, when naïve $CD4^+$ T cells from HIV-2+ patients were cultured in the presence of IL-7, their ability to proliferate in response to stimulation appeared to be well-preserved.

Methods

Study populations

Studied cohorts were specifically designed in order to include age-matched, untreated HIV-1+ and HIV-2+ individuals, in early stage disease (more than 300 $CD4^+$ T cells/μl), with no ongoing opportunistic infections or tumors. Table 1 depicts the epidemiological and clinical characteristics of the 11 HIV-2+ and 9 HIV-1+ individuals studied, as well as of the 9 age-matched seronegative controls. Written consent was

obtained for blood sampling and processing. The study was approved by the Ethical Board of the Faculty of Medicine of Lisbon.

Table 1. *Clinical and epidemiological data of the studied cohorts.*

	Seronegatives	**HIV-1**	**HIV-2**
Numbers (male/female)	9 (4/5)	9 (6/3)	11 (2/9)
Age (years)	43±3 (32-57)	46±4 (25-63)	52±3 (33-66)
Ethnicity (caucasian/other)	9/0	6/3	7/4
% CD4$^+$ T cells	45.6±3.1 (26.2-58.2)	25.4±3.3 (18.0-50.1)**	30.5±2.4 (16.8-45.7)**
CD4$^+$ T cells/µl	1114±103 (860-1649)	567±62 (300-939)***	766±125 (314-1464)
% Naïve CD4$^+$ T cells[a]	16.7±1.9 (7.1-24)	12.1±2.2 (7.3-27.6)	12.2±1.4 (6.2-22.1)
% HLA-DR$^+$CD38$^+$ in CD4	1.2±0.2 (0.5-2.4)	4.9±1.2 (0-9-11.3)*	2.7±0.6 (1.0-6.6)*
% HLA-DR$^+$CD38$^+$ in CD8	5.5±1.5 (1.5-12.9)	31.1±5.1 (13.5-54)***	15.8±2.7 (6.3-35.9)**/#
Viremia (RNA copies/ml)	NA	$3.3x10^4 \pm 10^4$ (295-$7.3x10^4$)	<200

Data are Mean±SEM with limits in brackets, unless indicated otherwise. NA, Not applicable. Statistical differences between a given HIV infected cohort and seronegative controls: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Statistical differences between HIV-1 and HIV-2 infected individuals: #, $p < 0.05$.
[a] Defined as the percentage of CD4$^+$CD45RA$^+$ cells, in a lymphocyte gate of whole PBMC, according to forward and side scatter characteristics, which were confirmed to be CD27$^+$ in all individuals.

PBMC isolation and naïve CD4$^+$ T-cell purification

Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinised venous blood by density gradient centrifugation on Ficoll-Paque PLUS (GE Healthcare, Sweden), as previously described [24]. Immunomagnetic separation of untouched naïve CD4$^+$ T cells from PBMC was performed, according to the manufacturer's instructions (Human Naïve CD4$^+$ T-cell Enrichment Kit, StemCell Technologies, France). Sorted naïve CD4$^+$ T-cell purity was assessed by flow cytometry within a lymphocyte gate, defined by forward and side scatter characteristics (mean

frequency of $CD4^+CD45RA^+$ cells was 95±1% for seronegatives, 92±1% for HIV-1+ individuals, and 94±1% for HIV-2+ patients).

Cell cultures

Purified naïve $CD4^+$ T cells were cultured at $1x10^6$ cell/ml in round-bottom 96-well plates in RPMI1640 supplemented with 100 U/ml penicillin/100 µg/ml streptomycin, 2 mM glutamine (all from Gibco-Invitrogen, U.K.) and 10% human AB serum (Sigma-Aldrich), in the presence of either IL-7 (10 ng/ml) or IL-2 (20 U/ml), at 37ºC with 5% CO_2, for seven days. Cells were re-fed with medium supplemented with the appropriate cytokine at the indicated concentration after three days of culture, and harvested on day 7 for subsequent flow cytometric analysis.

Flow cytometry

Surface and intracellular staining were performed as previously described [11, 24], using combinations of the following monoclonal antibodies (clone and manufacturer specified in brackets): FITC-conjugated CD4 (RPA-T4, eBiosciences, USA), CD45RA (HI100, eBiosciences, USA), HLA-DR (L243, BD Biosciences, USA), and Bcl-2 (124, Dako, Denmark); PE-conjugated CD38 (HB7, eBiosciences, USA), CD95 (DX2, eBiosciences, USA), CD127 (40131, R&D systems, USA) and Ki-67 (B56, BD Biosciences, USA); PerCP-conjugated CD3 (OKT3, eBiosciences, USA), CD4 (SK3, eBiosciences, USA), and CD45RA (HI100, eBiosciences, USA); PE-Cy7 CD27 (O323, eBiosciences, USA); APC-conjugated CD8 (RPA-T8, eBiosciences, USA) and CD31 (WM59, eBiosciences, USA); APC.Cy7-conjugated CD3 (SK7, BD Biosciences, USA), CD4 (RPA-T4, BD Biosciences, USA) and CD8 (SK1, BD Biosciences, USA). Briefly, for surface staining, cells were suspended in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA; Sigma-Aldrich) and 0.1% sodium azide (Sigma-Aldrich), stained with monoclonal antibodies (mAb) for 20 minutes in the dark at room temperature, extensively washed and fixed with 1% formaldehyde (Sigma-Aldrich). Intracellular staining for Bcl-2 and Ki-

67 was performed using fixation and permeabilization reagents from eBiosciences. Annexin V staining was performed using the Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences), according to the manufacturer's instructions, and acquisition was performed within one hour of the protocol's completion. Samples were acquired with a FACSCanto or a FACSCAlibur flow cytometer (both from BD Biosciences), and analyzed using FlowJo software (Tree Star, Ashland, OR). At least 50,000 or 100,000 events were acquired for each sample (on FACSCalibur or FACSCanto, respectively).

Serum IL-7 levels quantitation

IL-7 levels were assessed in serum samples, collected after centrifugation of freshly drawn blood and stored at -80ºC until use, with the commercially available Quantikine HS ELISA kit, according to the manufacturer's instructions (R&D systems). All samples were assayed in duplicate.

sjTREC quantification by real-time PCR

Isolated naïve $CD4^+$ T cells were lysed with proteinase K (Qiagen, Valencia, CA), in a proportion of 20 µl of proteinase K to $5x10^6$ cells, in order to obtain genomic DNA. Signal-joint TREC (sjTREC) were quantified by nested real-time PCR using Power SYBR Green PCR Master Mix (Applied Biosystems) and ABI PRISM 7500 FAST Sequence Detection System (Applied Biosystems). Specific primers and probes were used for sjTREC and CD3γ, used as a housekeeping gene for absolute quantification of sjTREC levels, as follows: sj-out5 5'-CTC TCC TAT CTC TGC TCT GAA-3'; sj-out3 5'-ACT CAC TTT TCC GAG GCT GA-3'; sj-in5 5'-CCT CTG TCA ACA AAG GTG AT-3'; sj-in3 5'-GTG CTG GCA TCA GAG TGT GT-3'; CD3-out5 5'-ACT GAC ATG GAA CAG GGG AAG-3'; CD3-out3 5'-CCA GCT CTG AAG TAG GGA ACA TAT-3'; CD3-in5 5'-GGC TAT CAT TCT TCT TCA AGG T-3'; CD3-in3 5'-CCT CTC TTC AGC CAT TTA AGT A-3'; sj-Probe1 5'-AAT AAG TTC AGC CCT CCA TGT CAC ACTf-3'; sj-Probe2 5'-XTG TTT TCC ATC CTG GGG AGT GTT TCAp-3'; CD3-Probe1 5'-GGC TGA AGG TTA GGG ATA CCA ATA TTC CTG TCT Cf-3'; CD3-Probe2 5'-XCT

AGT GAT GGG CTC TTC CCT TGA GCC CTT Cp-3'. The pCD3-TREC plasmid was kindly provided by Rémy Cheynier (Institute Cochin, Paris).

Plasma viral load and proviral DNA assessment

HIV-1 plasma viral load (viremia) was quantified by a reverse transcriptase PCR (RT-PCR) assay with a detection threshold of 40 RNA copies/ml (Roche, Basel, Switzerland). HIV-2 viremia was quantified using a previously described in-house-developed assay [25] with a detection threshold of 40 RNA copies/ml. The cut-off values of these tests were considered for statistical analysis, in cases where values were below these detection levels. Cell-associated HIV-1 and HIV-2 total viral DNA (integrated and nonintegrated viral DNA species) was quantified using real-time PCR assays that amplify highly conserved regions in HIV-1 and HIV-2 *gag*, with a detection range of 7 orders of magnitude and a sensitivity of 5 copies, as previously described [26].

Statistical analysis

Statistical analysis was performed using GraphPad Prism v5.00 (GraphPad Software Inc., San Diego, CA). Results are presented as arithmetic mean±SEM. Data were compared using Mann-Whitney or Wilcoxon tests, as appropriate. Spearman's correlation coefficient was used to determine association between two parameters. *P*-values <0.05 were considered significant.

Results

Ex-vivo analysis of naïve CD4$^+$ T cells in HIV-1+ and HIV-2+ individuals

In order to investigate naïve CD4$^+$ T-cell homeostasis, we studied HIV+ individuals in early stage disease as a means to avoid potentially confounding

immunopathologic factors associated with advanced HIV infection and to identify mechanisms that may help slow the rate of CD4$^+$ T-cell decline. In agreement with this strategy, recent data suggested that measuring peripheral naïve CD4$^+$ T cells from HIV+ individuals at earlier stages of disease may identify those at higher risk for poor CD4$^+$ T-cell reconstitution [27], supporting that even early disturbances in the naïve CD4$^+$ T-cell pool may impact on the rate of disease progression. Additionally, only the study of early stage HIV+ individuals would allow the isolation of sufficient naïve CD4$^+$ T cells from peripheral blood required to perform this type of study. Therefore, all HIV infected subjects had more than 300 CD4$^+$ T cells/µl and were therapy-naïve. Age-matched HIV-2 (n=11), HIV-1 (n=9), and seronegative (n=9) cohorts (Table 1) were studied to elucidate whether slower HIV-2 disease progression could be related to a better ability of HIV-2+ individuals to preserve their naïve CD4$^+$ T-cell pool.

Notwithstanding the distinct levels of viral load in HIV-2+ and HIV-1+ individuals (all undetectable viremia *vs.* all viremic, respectively), the two cohorts featured similarly increased levels of T-cell activation (Table 1). This is in agreement with our previous data showing that CD4$^+$ T-cell depletion is more directly associated with T-cell activation than viral load both in HIV-2 and HIV-1 infections [28]. Moreover, although HIV-2+ individuals were expected to have been infected for a much longer period of time than their HIV-1+ counterparts [18], the frequencies of total and naïve CD4$^+$ T cells were similar in both HIV cohorts (Table 1). However, HIV-1+ patients had significantly higher *ex vivo* frequencies of cycling naïve CD4$^+$ T cells (assessed by Ki-67 expression, Figures 1A and 1B), as compared to both age-matched HIV-2+ individuals and seronegative controls. Importantly, the specific contribution of the CD31$^+$ subset, which is enriched in RTE, to this pool of cycling naïve CD4$^+$ T cells was significantly lower in HIV-1+ than HIV-2+ individuals, the latter also presenting an increased proportion of cycling CD31$^+$ naïve CD4$^+$ T cells in relation to seronegative controls (Figure 1C). Of note, no significant differences were observed in circulating IL-7 levels in the two infected cohorts (HIV-1+, n=9: 16.61±1.65 pg/ml; HIV-2+, n=10: 13.62±1.60 pg/ml).

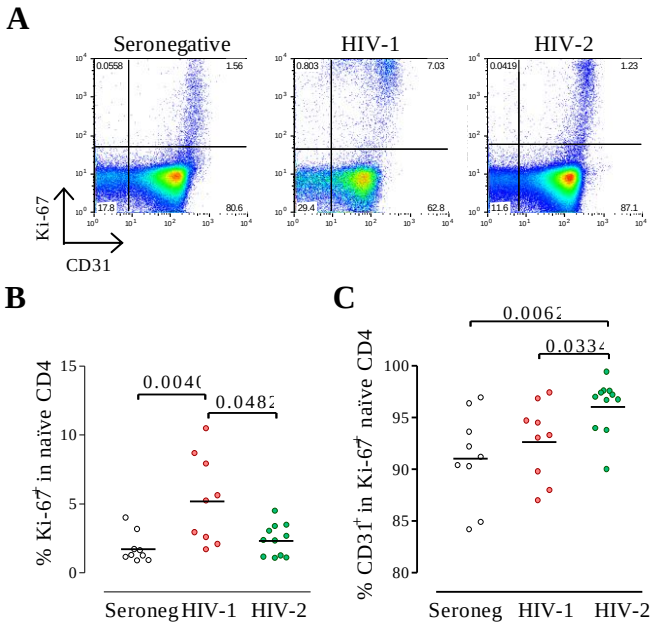

Figure 1. *Ex vivo* frequency of cycling naïve CD4$^+$ T cells in early HIV-2 and HIV-1 infection. (A) Illustrative dot-plots of the expression of the cell cycling marker Ki-67 and of CD31 expression on gated naïve CD4$^+$ T cells analysed *ex vivo*. Representative HIV+ individuals presented similar CD4$^+$ T-cell counts (HIV-1: 546 CD4$^+$ T cells/µl; HIV-2: 635 CD4$^+$ T cells/µl), but distinct viremia (HIV-1: 49 918 RNA copies/ml; HIV-2: undetectable). **(B)** Frequency of Ki-67$^+$ cells within total naïve CD4$^+$ T cells in HIV-2, HIV-1 and seronegative cohorts. **(C)** Frequency of CD31$^+$ cells within Ki-67$^+$ naïve CD4$^+$ T cells in the same cohorts. Each dot represents one individual, bars represent mean. Data were compared using Mann-Whitney tests. *P*-values <0.05 were considered significant and are indicated.

Overall, these results suggest that, despite a similar frequency of naïve CD4$^+$ T cells in early stage HIV-1 and HIV-2 infection, the mechanisms underlying the maintenance of the naïve CD4$^+$ T-cell pool appear distinct. Whilst HIV-1+ patients apparently relied on extensive cell cycling, including that of the CD31$^-$ subset, in HIV-2 disease a preferential proliferation of RTE-enriched CD31$^+$ cells, likely mediated by IL-7, in association with the expected better preserved thymic output [29], contributed to naïve CD4$^+$ T-cell preservation.

Naïve CD4$^+$ T cells from HIV-2+ individuals featured a better preserved proliferative response to IL-7 upon in vitro stimulation

To elucidate the role of IL-7-induced proliferation in the maintenance of naïve CD4$^+$ T cells during HIV infection, these cells were isolated from early stage HIV-1+ and

HIV-2+ patients, and seronegative controls, and cultured for seven days in the presence of IL-7. In parallel, cells were cultured with IL-2 as a control, given the high rates of purified naïve CD4+ T-cell death in culture without cytokines. Cells were harvested on day 7, and flow cytometry analysis confirmed the maintenance of a naïve phenotype throughout culture both with IL-7 and IL-2. Cell recovery of live cells, assessed by direct cell count *via* trypan blue dye exclusion, was higher for cells cultured in the presence of IL-7 in all cohorts (data not shown), in agreement with previous reports [11].

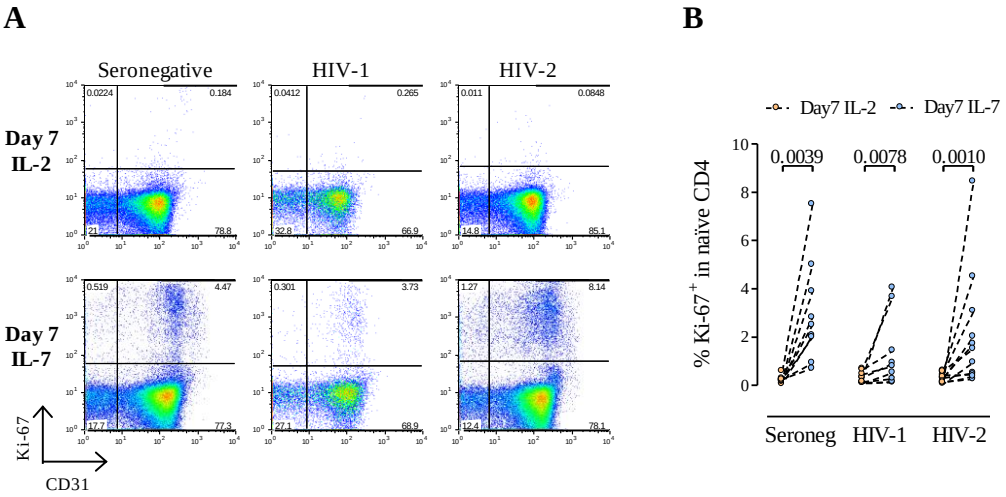

Figure 2. Ability of naïve CD4+ T cells to proliferate *in vitro* in response to IL-7 stimulation in early HIV-2 and HIV-1 infection. (A) Illustrative dot-plots of the expression of the cell cycling marker Ki-67 and of CD31 expression on cultured naïve CD4+ T cells. Representative HIV+ individuals presented similar CD4+ T-cell counts (HIV-1: 546 CD4+ T cells/µl; HIV-2: 635 CD4+ T cells/µl), but distinct viremia (HIV-1: 49 918 RNA copies/ml; HIV-2: undetectable), at the time of sampling. **(B)** Frequency of Ki-67+ cells within total naïve CD4+ T cells upon 7 days of culture in the presence of IL-7 (black dots) or IL-2 (grey) in HIV-2, HIV-1 and seronegative cohorts. Each pair of connected grey and black dots corresponds to one individual. Inter- and intra-cohort data were compared using Mann-Whitney or Wilcoxon tests, respectively. *P*-values <0.05 were considered significant and are indicated.

As illustrated in Figure 2, naïve CD4+ T cells from HIV-2+ individuals featured a preserved IL-7 responsiveness in terms of cycling, which was similar to that of age-matched seronegatives. In contrast, naïve CD4+ T cells from HIV-1+ individuals featured an overall decreased ability to expand in response to IL-7 in comparison to seronegative controls (p=0.0503). Notably, the distinct ability to respond upon *in vitro* IL-7 stimulation was not apparently related to the basal levels of IL-7Rα (CD127)

expression on naïve $CD4^+$ T cells, since these were not significantly different in the two infected cohorts with early stage disease (MFI of CD127: 78.62±1.26, n=9 HIV-1+ subjects; 75.32±4.02, n=10 HIV-2+ subjects).

These findings suggest a better preserved response to γc cytokines in the context of HIV-2 infection, which may be related to the slower $CD4^+$ T-cell depletion and more "benign" disease course that characterize this infection.

Similar impact of IL-7 stimulation on anti- and pro- apoptotic molecules in naïve $CD4^+$ T cells from HIV-2+ and HIV-1+ individuals

One of the main functions of IL-7 is the promotion of cell survival through the up-regulation of the anti-apoptotic molecule Bcl-2 [30]. Nevertheless, IL-7 has also been shown to up-regulate Fas (CD95) expression and, in this way, promote cell death [31, 32]. In order to further investigate these processes in the context of early stage HIV disease, the expression of Bcl-2 and CD95 was assessed.

As shown in Figure 3A (left and middle graphs, respectively), although no significant inter-cohort differences were observed in the *ex vivo* expression of Bcl-2, the frequency of CD95-expressing naïve $CD4^+$ T cells tended to be increased in both HIV cohorts, although only reaching significance relative to age-matched seronegatives for HIV-1+ individuals. Of note, the *ex vivo* balance of anti- *vs.* pro-apoptotic factors resulted in only a borderline increase in the overall frequency of early apoptotic and dead naïve $CD4^+$ cells, evaluated as the percentage of Annexin V^+ cells, in the HIV-1 cohort (Figure 3A, right-hand graph), which did not reach statistical significance.

All cohorts showed a preserved ability to up-regulate Bcl-2 and CD95 expression on naïve $CD4^+$ T cells upon IL-7 stimulation (Figure 3B, left and middle graphs, respectively). Moreover, there were no differences in the overall frequency of Annexin V expression (Figure 3B, graph on the right) which was, as expected,

significantly lower than that observed for naïve CD4+ T cells cultured in the presence of IL-2.

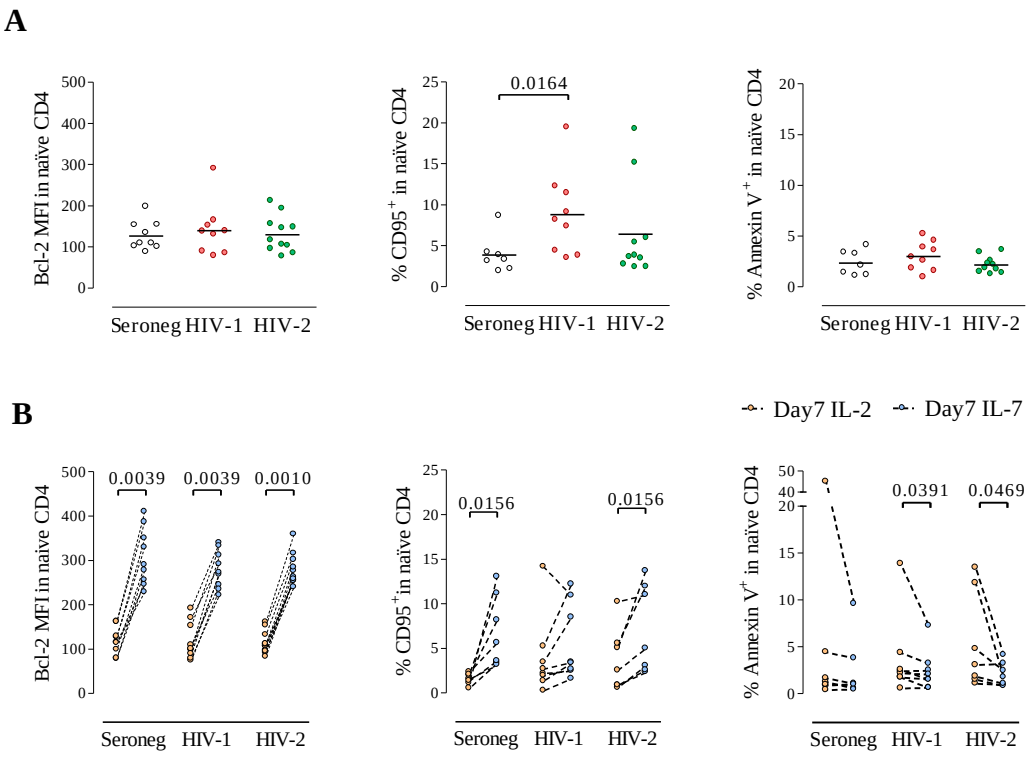

Figure 3. *Ex vivo* expression of anti- and pro-apoptotic molecules by naïve CD4+ T cells and up-regulation upon IL-7 stimulation in early HIV-2 and HIV-1 infection. Purified naïve CD4+ T cells were analysed by flow cytometry, both *ex vivo* **(A)**, and after 7-day culture with either IL-7 or IL-2, as a control **(B)**. Naïve cells were characterized in terms of Bcl-2 (graphs on the left) and CD95 expression (graphs in the middle). Cell death and commitment to undergo apoptosis was estimated by the total percentage of Annexin V+ cells (graphs on the right). Each open dot represents one individual; bars represent mean. Closed dots represent individuals whose cells were cultured in the presence of IL-7 (black) or IL-2 (grey). Each pair of connected grey and black dots corresponds to one individual. Inter- and intra-cohort data were compared using Mann-Whitney or Wilcoxon tests, respectively. *P*-values <0.05 were considered significant and are indicated.

Taken together, our results suggest that discrepancies in IL-7 responsiveness of naïve CD4+ T cells from HIV-2 and HIV-1 cohorts were mainly related to proliferation. Our data further support that distinct intracellular signaling pathways are involved in IL-7-induced proliferation and up-regulation of anti- and pro-apoptotic factors, which are differently affected in HIV-2- and HIV-1-infected individuals.

Naïve CD4$^+$ T cells from HIV-2+ individuals showed preserved levels of CD31 expression and were able to up-regulate CD31 upon IL-7 stimulation

We have previously shown that CD31$^+$ naïve CD4$^+$ T cells from adults preferentially expand in response to IL-7 without a concomitant loss of CD31 expression, potentially contributing to the maintenance of the naïve CD4$^+$ T-cell compartment size and diversity [11].

As mentioned above, the contribution of the CD31$^+$ subset to the overall pool of cycling naïve CD4$^+$ T cells was found to be significantly increased in the context of HIV-2 infection, both in relation to age-matched HIV-1+ and seronegative individuals (Figure 1C). This was despite the lack of statistically significant differences observed in terms of the *ex vivo* frequencies of CD31$^+$ cells within total naïve CD4$^+$ T cells in both cohorts (Figure 4A). Nevertheless, when the density of CD31 molecules *per* cell (assessed as Mean Fluorescence Intensity, MFI) was evaluated within the CD31$^+$ naïve CD4$^+$ T-cell population, significantly lower levels were found in HIV-1+ patients, as compared to both HIV-2+ and seronegative individuals (Figure 4B). This may in part be related to the significant reduction in CD31^{bright} cell frequency within the naïve CD4$^+$ T-cell pool observed in HIV-1+ (22.4±2.3%; p=0.040), but not HIV-2+ individuals (30.9±2.5%; p=0.790), in comparison to age-matched seronegative controls (31.1±3.3%). As it has been demonstrated that the CD31^{bright} naïve CD4$^+$ T-cell subset includes the majority of RTE [33], our observations may indicate impaired thymic output even in early HIV-1 disease. RTE are known to express high levels of sjTRECS [29]. We quantified sjTREC levels in purified naïve CD4$^+$ T cells, and found no significant inter-cohort differences (seronegatives: 2261±741; HIV-1+: 1463±353; HIV-2+: 1508±475 sjTREC/10^5 naïve CD4$^+$ T cells). Of note, no correlations were found between sjTREC levels and CD31^{bright} frequency in naïve CD4$^+$ T cells in any cohort (data not shown). However, we observed significant positive correlations between sjTREC levels in naïve CD4$^+$ T cells and both the percentage of circulating naïve CD4$^+$ T cells and the frequency of CD31$^+$ cells within naïve CD4$^+$ T cells in HIV-1+ patients (r=0.7167, p=0.0298; and r=0.8000, p=0.0096, respectively), suggesting a possible

preferential incorporation of RTE in the peripheral naïve CD4$^+$ T-cell pool in the context of HIV-1 infection.

Thus, despite the evidence of an apparent incorporation of RTE in the naïve CD4$^+$ T-cell pool in HIV-1+ individuals, their levels of CD31 were significantly reduced, suggesting an impaired *in vivo* response to IL-7, which was not observed in early HIV-2 infection.

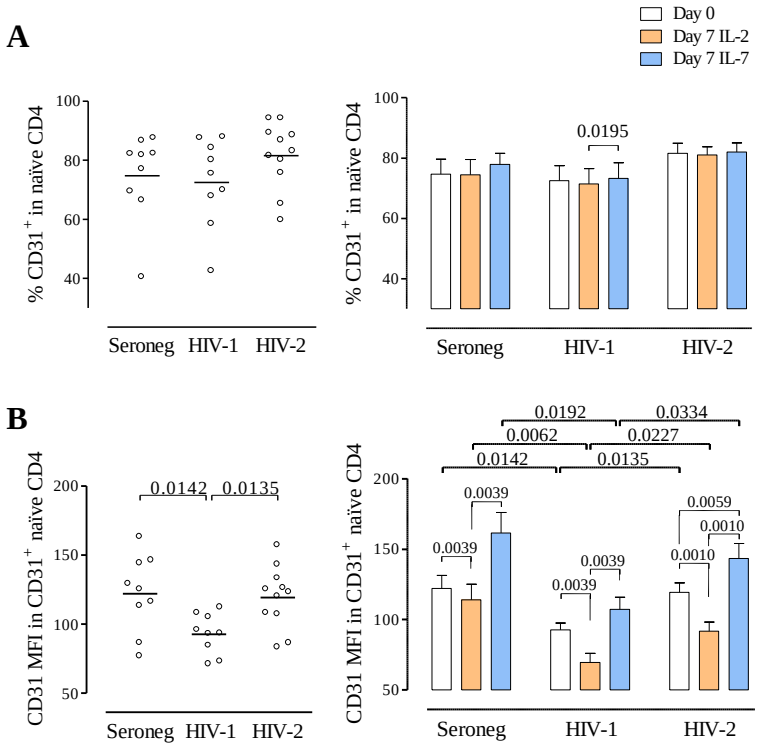

Figure 4. The CD31$^+$ subset of naïve CD4$^+$ T cells in early HIV-2 and HIV-1 infection. Purified naïve CD4$^+$ T cells characterized in terms of the relative frequency of CD31$^+$ cells **(A)** and the amount of CD31 expression *per* CD31$^+$ cell (assessed as mean fluorescence intensity, MFI) **(B)**. Graphs on the left refer to the assessment of these parameters *ex vivo,* with those on the right indicating their variation from the basal values (white bars) upon 7-day stimulation with either IL-7 (black bars), or IL-2 (grey bars). Each dot represents one individual; bars represent mean. Inter- and intra-cohort data were compared using Mann-Whitney or Wilcoxon tests, respectively. *P*-values <0.05 were considered significant and are indicated.

Our group has previously reported that IL-7 increases the levels of CD31 expression on CD31$^+$ naïve CD4$^+$ T cells from healthy subjects, without inducing its expression on their CD31$^-$ counterparts [11]. We found a statistically significant increase in the levels of CD31 expression within CD31$^+$ cells upon IL-7 stimulation in HIV-2+ patients (Figures 4A and 4B).

Overall, our data supported a maintained response to IL-7 *in vivo* and *in vitro* in HIV-2 infection.

Limited contribution of IL-7 to the extensive cycling of naïve CD4$^+$ T cells observed in early HIV-1 infection

We have previously shown that HIV-2+, as compared to HIV-1+ individuals, have better preserved thymic function [29], despite the fact that they have, most-likely, been infected for a longer period of time [18]. Thus, it is possible that these individuals preferentially compensate for naïve CD4$^+$ T-cell loss through increased CD4$^+$ T-cell production and/or maintenance of RTE cells within the naïve CD4$^+$ T-cell pool; whereas naïve CD4$^+$ T-cell homeostasis in HIV-1+ patients may be more reliant on extensive peripheral expansion. A concomitant and pronounced loss of the CD31^{bright} subset would suggest this extensive proliferation to be mainly IL-7-indpendant.

In agreement with this hypothesis, HIV-1+ patients had significantly higher *ex vivo* frequencies of cycling CD31$^+$ and CD31$^-$ naïve CD4$^+$ T cells, as compared to HIV-2+ individuals (Figures 5A and 5B, respectively). Furthermore, direct correlations were found between the cycling of CD31$^-$ naïve CD4$^+$ T cells and both viremia (r=0.7500, p=0.0199) and T-cell activation (CD4: r=0.8000, p=0.0096; CD8: r=0.7833; p=0.0125) in HIV-1 infection.

These data suggest a marked loss of CD31 expression during extensive proliferation of naïve CD4$^+$ T cells, as well as additionally increased proliferation/differentiation of the CD31$^-$ subset, probably *via* self-peptides-MHC stimulation, in the context of HIV-1, but not HIV-2 infection. Of note, proviral DNA in purified naïve CD4$^+$ T cells was only detectable in HIV-1+ individuals (270±51 copies/10^6 naïve CD4$^+$ T cells, n=3 out of 9 patients).

On the other hand, only HIV-2+ individuals featured a direct correlation between naïve CD4$^+$ T-cell cycling and CD31 MFI within the CD31$^+$ population

(r=0.7545, p=0.0073), suggesting that this particular, RTE-enriched, subset is relevant for the maintenance of naïve CD4$^+$ T cells in this infection, probably through IL-7-induced homeostatic proliferation.

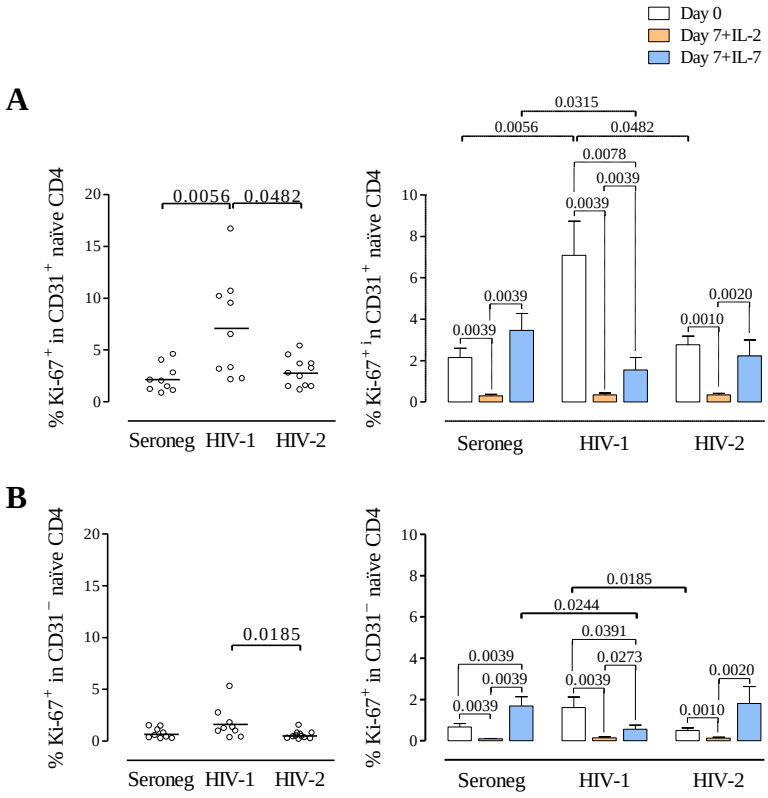

Figure 5. Cell cycling of naïve CD4$^+$ T-cell subsets defined according to CD31 expression in early HIV-2 and HIV-1 infection. Purified naïve CD4$^+$ T cells characterized in terms of the relative frequency of cycling cells (assessed as those expressing Ki-67$^+$) within the CD31$^+$ **(A)** and CD31$^-$ **(B)** subsets. Graphs on the left refer to this characterization *ex vivo,* with those on the right indicating their variation from the basal values (white bars) upon 7-day stimulation with either IL-7 (black bars), or IL-2 (grey bars). Each dot represents one individual; bars represent mean. Inter- and intra-cohort data were compared using Mann-Whitney or Wilcoxon tests, respectively. *P*-values <0.05 were considered significant and are indicated.

Importantly, although some degree of proliferation was observed in CD31$^-$ cells in response to IL-7, the CD31$^+$ subset was the one found to preferentially cycle in response to this cytokine in all cohorts. However, this capacity was significantly reduced in HIV-1+ patients in relation to seronegative controls, whilst being maintained in the HIV-2 cohort (Figures 5A and 5B). Of note, both CD31$^+$ and CD31$^-$ naïve CD4$^+$ T-cell subsets featured similar up-regulation of Bcl-2 and CD95 in all cohorts (Figure 6), supporting the impact of IL-7 on survival/apoptotic pathways in

HIV+ patients being irrespective of CD31 expression, as previously reported in healthy subjects [11].

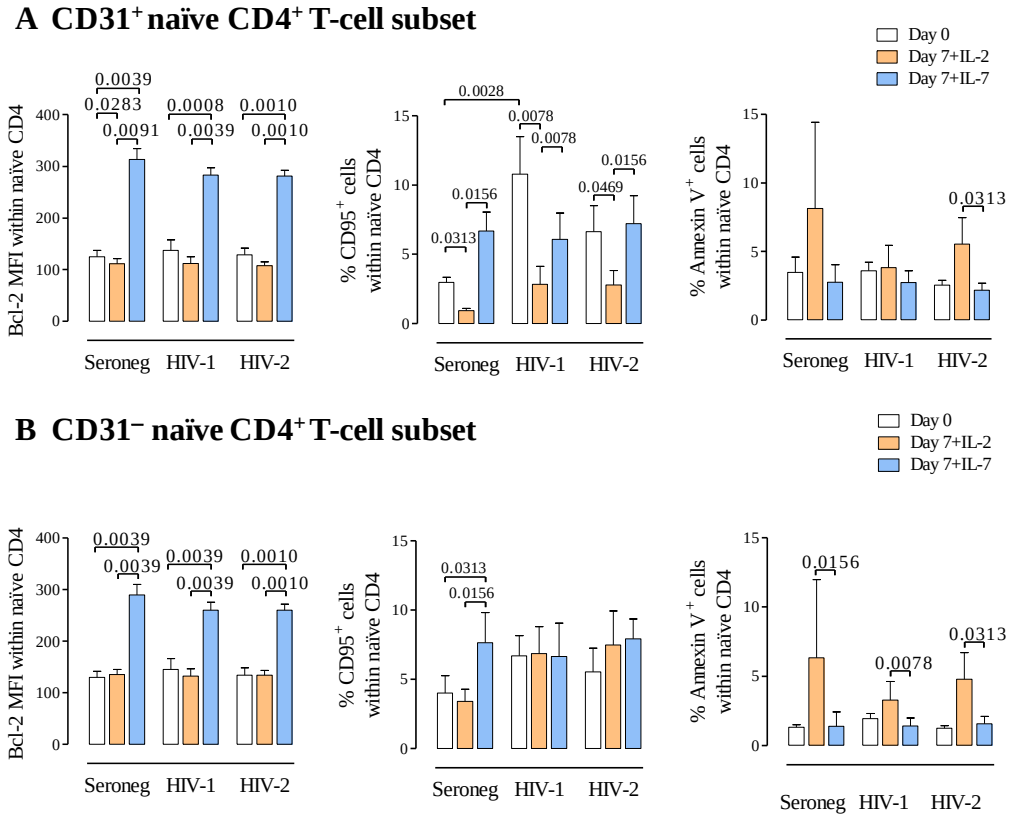

Figure 6. Expression of anti- and pro-apoptotic molecules in naïve CD4$^+$ T cell subsets defined according to CD31 expression in early HIV-2 and HIV-1 infection. Purified naïve CD4$^+$ T cells were gated according to CD31 expression: CD31$^+$ **(A)** and CD31$^-$ **(B)**; and characterized in terms of Bcl-2 (graphs on the left) and CD95 expression (graphs in the middle). Cell death and commitment to undergo apoptosis was estimated by the total percentage of Annexin V$^+$ cells (graphs on the right). Graphs refer to the evaluation of these parameters both *ex vivo* (white bars), and after 7-day stimulation with either IL-7 (black bars), or IL-2 (grey bars). Due to cell availability, CD95 expression and Annexin V$^+$ cell frequency were only assessed in sub-groups of age-matched individuals (7/9 seronegatives; 9/9 HIV-1+ and 10/11 HIV-2+ for the *ex vivo* characterization; 7/9 seronegatives; 8/9 HIV-1+ and 7/11 HIV-2+ for the analysis upon culture in the presence of cytokines). Bars represent mean±SEM. Inter- and intra-cohort data were compared using Mann-Whitney or Wilcoxon tests, respectively. *P*-values <0.05 were considered significant and are indicated.

Our results suggest that, in HIV-2 infection, naïve CD4$^+$ T-cell homeostasis was maintained by a combination of preserved thymic output [29] and the preferential IL-7-mediated homeostatic proliferation of CD31$^+$ naïve CD4$^+$ T-cell subset. Conversely, in HIV-1 infection, the maintenance of the naïve CD4$^+$ T-cell compartment appeared to rely on extensive peripheral proliferation, with a significant contribution of the CD31$^-$

subset, probably *via* self-peptides-MHC stimulation, although a direct contribution of HIV-1 antigenemia cannot be excluded.

Discussion

We found that distinct mechanisms underpinned the maintenance of naïve CD4$^+$ T cells in early stage HIV-1 and HIV-2 infections. HIV-2+ patients featured a significant over-representation of RTE-enriched CD31$^+$ cells within the *ex vivo* pool of cycling naïve CD4$^+$ T cells, both relative to seronegative and HIV-1+ individuals. Of note, as this occured in the presence of sustained CD31 expression levels, it suggested a role for IL-7, given its ability to induce the proliferation of the CD31$^+$ subset and to sustain/increase CD31 expression [11]. The HIV-1 cohort featured a significantly higher frequency of cycling naïve CD4$^+$ T cells, with a significant involvement of the CD31$^-$ subset. These *ex-vivo* data support distinct abilities to respond to IL-7 in the two infections, which were confirmed *in vitro* in cultures of purified naïve CD4$^+$ T cells with IL-7 or IL-2. Naïve CD4$^+$ T cells from HIV-2+ individuals were better able to proliferate and to up-regulate CD31 upon *in vitro* stimulation with IL-7. Of note, a similar impact of IL-7 stimulation on anti- and pro- apoptotic molecules were observed in naïve CD4$^+$ T cells from HIV-2+ and HIV-1+ individuals.

We have previously shown that IL-7 preferentially expands the CD31$^+$ naïve CD4$^+$ T-cell subset, without concomitant loss of CD31 expression [11], thus helping maintain the size and diversity of the naïve CD4$^+$ T-cell pool during adulthood. Moreover, our previous report of a delayed increase in IL-7 levels, and a better sustained IL-7Rα chain expression, particularly in the naïve CD4$^+$ T-cell pool, during HIV-2 disease progression [21], suggested that HIV-2+ individuals may have a sustained ability to respond upon IL-7 stimulation. In agreement with this possibility, only HIV-2+, and not HIV-1+ patients, featured preserved proliferative responses of naïve CD4$^+$ T cells, and in particular of the CD31$^+$ subset, to IL-7 stimulation in relation to age-matched seronegative controls.

Of note, the studied cohorts showed no differences in naïve $CD4^+$ T-cell ability to up-regulate anti- and pro-apoptotic factor expression upon IL-7 stimulation.

After binding to its receptor, IL-7 signals via both Jak/STAT5 and PI3K/Akt pathways, with STAT5 acting both directly as a transcription factor, and indirectly by triggering additional PI3K pathway-dependent signaling [34]. The two pathways interact to promote T-cell survival and proliferation [30, 35]. Nevertheless, a number of studies have provided evidence that IL-7-induced proliferation is critically dependent on PI3K activity [11, 35, 36]. Defects in IL-7 responsiveness due to disturbances in the Jak/STAT5 signaling pathway have been extensively reported in HIV-1 infection [14-16, 37, 38]. As the survival and proliferation effects of IL-7 on human RTEs have been shown to be distinguished on the basis of dose [35], it is possible that STAT5 signaling impairment, and consequent PI3K under-stimulation, in HIV-1 disease, translate into lower levels of IL-7-mediated signaling. This may help explain our data indicating that discrepancies in naïve $CD4^+$ T cell IL-7 responsiveness in the HIV-2 and HIV-1 cohorts were mainly related to proliferation. Hence, the study of how distinct intracellular pathways involved in IL-7 responsiveness are affected during HIV-2 and HIV-1 disease is a subject warranting further investigation. Taken together, our results suggest that, in early HIV-2 infection, preservation of the naïve $CD4^+$ T-cell pool largely relies on the maintenance of an RTE-enriched population in the periphery *via* preferential IL-7-mediated homeostatic proliferation.

On the other hand, in early HIV-1 infection, the high levels of viremia, and thus large amounts of circulating antigen, potentially cause increased pressure on the naïve $CD4^+$ T-cell compartment, forcing naïve cells to enter the memory pool at a higher rate.

Nevertheless, as previously mentioned, comparable frequencies of naïve $CD4^+$ T cells were documented for HIV-1 and HIV-2 infections. Moreover, we found no significant differences in the naïve $CD4^+$ T cell sjTREC levels between cohorts, despite the higher levels of cycling reported in HIV-1 infection. As the measurement of sjTREC levels is known to be affected by their progressive dilution during peripheral cell proliferation [39], our observations may indicate a preferential incorporation of RTE

(with high sjTREC content) in the peripheral naïve CD4$^+$ T-cell pool in the context of HIV-1 infection, which will then help sustain the naïve CD4$^+$ T-cell pool via extensive proliferation. In agreement, significant positive correlations between sjTREC levels in naïve CD4$^+$ T cells and both the percentage of circulating naïve CD4$^+$ T cells and the frequency of CD31$^+$ cells within naïve CD4$^+$ T cells were exclusive to the HIV-1 cohort. The dynamics of human RTE incorporation into the peripheral naïve T-cell pool remains unclear [5]. However, it is possible that, in agreement with recently published data [40], new CD4$^+$ T cells are preferentially incorporated into the depleted peripheral naïve CD4$^+$ T-cell pool in HIV-1+ infection increasing the average sjTREC content even in the presence of a reduction in thymic output [41, 42]. In the future it will be relevant to investigate possible reasons for a putative lower rate of incorporation of RTE in HIV-2+ individuals. Data from our group showed that, HIV-2+ but not HIV-1+ individuals were able to maintain thymic production, as assessed by the ratio of sj/βTREC, for prolonged periods of time, even after 45 years of age comparable levels of sjTREC were found in the two infections [29]. Importantly, although thymic output was not formally evaluated in our HIV-1 cohort, the significantly reduced frequencies of CD31^{bright} naïve CD4$^+$ T cells that were observed in these individuals, argue against it playing a major role in naïve CD4$^+$ T-cell maintenance.

In contrast to HIV-2 infection, naïve CD4$^+$ T-cell homeostasis in early HIV-1 infection seemed to be highly dependent on the extensive proliferation of CD31$^-$, and not CD31$^+$, cells. It is likely that this increased cell cycling results from low-affinity self-peptide-MHC interactions, a process that can result in the loss of CD31 expression and, thus, the preferential expansion of the CD31$^-$ naïve T-cell subset. Of note, this observation is in agreement with previous data showing that, in the context of thymic impairment, CD31$^+$ cells decreased, whilst the CD31$^-$ subset was maintained via extensive proliferation [43]. Additionally, the increased self-peptide/MHC-induced proliferation may be a consequence of the impaired ability to proliferate in response to IL-7. These results are particularly relevant given the recently proposed use of IL-7 therapy in HIV-1 infection [44-46].

Overall, we found a better preserved proliferative response of naïve $CD4^+$ T cells to IL-7 stimulation in HIV-2 infection, which could contribute to its characteristically slow disease progression. Further investigation of the mechanisms underlying the distinct response to IL-7 in the two HIV infections may help in the development of new therapeutic strategies aimed at achieving immunological competence in HIV-1 infection.

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3.5. Memory B-cell depletion is a feature of HIV-2 infection even in the absence of detectable viremia

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Abstract

Objective: Memory B-cell loss has long been recognized as an important contributor to HIV immunodeficiency. HIV-2 infection, which is characterized by a slow rate of progression to AIDS and reduced to undetectable viremia, provides a unique model to investigate B-cell disturbances.

Design and Methods: B-cell subsets were evaluated in 38 HIV-2-infected individuals, along with markers of T-cell activation and serum levels of Immunoglobulins and a major B-cell homeostatic cytokine, B-cell Activating Factor (BAFF). Untreated HIV-1-infected and non-infected control subjects were studied in parallel. Statistical analysis was performed using Mann-Whitney tests and Spearman's correlations.

Results: We found that HIV-2 was associated with significant depletion of both unswitched ($CD27^{+}IgD^{+}$) and switched ($CD27^{+}IgD^{-}$) memory B cells that directly correlated with T-cell activation, even in individuals with undetectable plasma viral load. Nevertheless, the presence of detectable viremia, even at low levels, was associated with significant memory B-cell loss and higher BAFF levels. Moreover, these alterations were not recovered by antiretroviral-therapy, as treated HIV-2+ patients featured more pronounced B-cell disturbances, possibly related to their extended length of infection.

Conclusions: These first data regarding B-cell imbalances during HIV-2 infection show that, irrespective of viremia, prolonged HIV infection leads to irreversible damage of memory B-cell homeostasis.

Introduction

HIV-1 infection is associated with progressive impairment of specific humoral responses and loss of memory B cells that are only partly recovered by antiretroviral therapy (ART) [1-4]. These B-cell disturbances have been linked to the persistent heightened state of immune activation associated with HIV-1 infection [3-5]. In fact, polyclonal B-cell activation with marked hypergammaglobulinemia is usually observed in acute HIV-1 infection [6], persisting throughout the chronic phase of the disease [3, 5]. HIV-1 proteins, particularly gp120 [7, 8] and Nef [9, 10], have also been shown to induce intrinsic B-cell functional defects.

HIV-2 infection is characterized by a much slower rate of disease progression, with low to undetectable viremia, providing a unique naturally-occurring model of attenuated disease to investigate B-cell disturbances in HIV pathogenesis. HIV-2 plasma viral load is usually low to undetectable throughout the entire disease course [11, 12]. This low viremia is thought to account for the low transmission rates [13, 14], contributing to the confinement of HIV-2 infection to West Africa and connected countries, such as Portugal [15, 16]. Additionally, HIV-2 has limited impact on the mortality of infected adults, even in rural West African areas, where its prevalence has reached 8-10% [17-19]. In agreement, a prospective study of a French HIV-2 cohort has revealed that the rate of $CD4^+$ T-cell decline is, on average, 10 times lower than in HIV-1+ individuals, leading to a median time of progression to AIDS of more than 20 years [20].

We have previously shown that, as for HIV-1, $CD4^+$ T-cell depletion is directly linked to increased immune activation in HIV-2+ individuals [12, 21]. Moreover, HIV-2 infection also induces polyclonal B-cell activation, as demonstrated by the frequently observed hypergammaglobulinemia [22, 23] and hyperplastic lymph nodes [24]. Of note, several studies have suggested a better ability of HIV-2+, in comparison with HIV-1+ individuals, to generate and preserve significant levels of circulating HIV-neutralizing antibodies during the chronic phase of the disease [23, 25-28]. This finding is thought to be mainly related to particular conformations of the HIV-2 envelope proteins that favour triggering of potent neutralizing antibody responses

[23, 25-30], although the possibility of a superior function of the B-cell compartment in HIV-2+ patients has not been formally evaluated.

Here we report the first data on memory B-cell imbalances during HIV-2 infection. We found an unexpected major depletion of both switched and unswitched memory B cells not recovered by ART in HIV-2+ individuals, suggesting that, irrespective of viremia, prolonged HIV infection leads to irreversible damage of memory B-cell homeostasis.

Methods

Studied cohorts

The study involved 38 HIV-2+, 20 HIV-1+, and 16 non-infected (Seronegatives) individuals. Table 1 details the cohort clinical and epidemiological data. HIV+ patients were followed at the Hospital de Santa Maria, Lisbon, and had no ongoing opportunistic infections or tumours. All patients gave written informed consent for blood sample collection and processing. The study was approved by the Ethical Board of the Faculty of Medicine, University of Lisbon. All HIV-1+ individuals were therapy-naive. The HIV-2 cohort included 10 patients on ART that did not differ significantly from the untreated HIV-2+ subjects in respect to viremia and proviral DNA levels (Supplemental Table 1). HIV-2+ individuals on ART showed significantly reduced $CD4^+$ T cells, both in relation to seronegatives and untreated HIV-2+ patients (Supplemental Table 1), in agreement with previous reports on weak virologic and immunological responses to ART in HIV-2+ individuals [20, 31].

Plasma viral load and proviral DNA assessment

HIV viremia was quantified by RT-PCR for both HIV-1+ (detection threshold: 40 RNA copies/ml, Roche, Basel, Switzerland) and HIV-2+ (detection threshold: 200 RNA

copies/ml, as described [31]) individuals. Viremia was, as expected [12, 20, 32], significantly lower in HIV-2+ than in untreated HIV-1+ patients (Table 1).

HIV-1 and HIV-2 total viral DNA (integrated and non-integrated DNA species) were quantified, as previously described [33]. Briefly, DNA was extracted from $5x10^6$ PBMC and total viral DNA was quantified using real-time PCR assays amplifying highly conserved regions in HIV-1 and HIV-2 *gag* (detection range: 7 orders of magnitude; sensitivity: 5 copies). Cut-off values of the tests were considered for statistical analysis in cases where detection was below these levels.

Table 1. *Clinical and epidemiological characteristics of the studied cohorts.*

	Seronegatives[a]	**HIV-2**[a]	**HIV-1**[a]
Number [male/female]	16 [6/10]	38 [14/24]	20 [15/5]
Age, years	44 (27-57)	56 (19-78)$^{*/\#}$	38 (23-61)
Caucasian/black	15/1	21/17	15/5
CD4$^+$ T cells, %	59 (40-77)	32 (4-66)***	30 (2-74)***
CD4$^+$ T cells/µl	818 (518-1312)	470 (52-1511)**	358 (18-1848)*
Viremia, HIV RNA cp/ml	-	200 (200-34x10^3)$^{\#\#}$	15x10^3 (40-45x10^5)
Proviral DNA, cp/10^6 PBMC	-	78 (5-1033)	66 (5-975)
B cells, %	6 (3-13)	7 (3-20)	6 (2-16)
B cells/µl	120 (63-369)	133 (30-369)	118 (33-406)

Data are expressed as median, with limits in brackets. Statistical analysis was performed with Mann-Whitney tests. $^{*}P< 0.05$; $^{**}P< 0.01$; $^{***}P< 0.001$ in comparison with seronegatives. $^{\#}P< 0.05$; $^{\#\#}P< 0.01$ for comparisons between infected cohorts.
[a]A distinct cohort was used for serum BAFF quantification, as described in Figure 3.

Flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated immediately after venopuncture by Ficoll-Hypaque density gradient centrifugation (Sigma, St. Louis, MO, USA), and surface stained as previously described [34]. Briefly, after isolation, PBMC

were washed and surface-stained for 20 minutes at room temperature with the following monoclonal antibodies (mAb) (clone specified in brackets): FITC-conjugated CD10 (CB-CALLA), PE-Cy7-conjugated CD19 (HIB19), and APC-conjugated CD27 (O323) from eBioscience (San Diego, CA), as well as with PE-conjugated IgD (IA6-2) and APC-Cy7-conjugated CD3 (SK7) from BD Biosciences (San Jose, CA). At least 150,000 events were acquired using a CANTO flow cytometer (BD Biosciences) and analysed using FlowJo software (version 8.5.3, TreeStar, Inc., Ashland, OR). Cells were successively gated on lymphocytes, according to forward/side scatter characteristics, and B cells ($CD19^{+}CD3^{-}$), which were then analysed in terms of CD27 and IgD expression, as illustrated in Figure 1. T-cell activation was assessed as previously described [34], using the following mAbs (clone specified in brackets): FITC-conjugated HLA-DR (L243), PerCP-conjugated CD4 (SK3) and APC-Cy7- conjugated CD3 (SK7) from BD Biosciences; and PE-conjugated CD38 (HIT2) and APC-conjugated CD8 (RPA-T8), both from eBioscience.

IL-7, BAFF, total serum immunoglobulin, β2-microglobulin, and specific antibodies quantifications

Serum IL-7 and BAFF were quantified by ELISA (R&D Systems, Minneapolis, MN), according to the manufacturer's specifications. Samples were assayed in duplicate.

Total IgG, IgA and IgM were quantified by immunonephelometry (Beckman-Coulter, Brea, CA), and β2-microglobulin by immunoturbidimetry (Roche Diagnostics, Indianapolis, IN), at the clinical laboratory of the Hospital de Santa Maria.

Quantification of specific antibodies against HIV-2 *env* glycoproteins gp36 and gp125 (C2-C3 region) was performed using a dual-antigen ELISA, as previously described [29]. The results of the assay were expressed quantitatively as $OD_{clinical\ sample}/OD_{cut\text{-}off}$ (S/CO) ratios. For ratio values of >1, the samples were considered seroreactive.

Statistical analysis

Statistical analysis was performed with Mann-Whitney tests and Spearman's correlations using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA). Results were expressed as median and *P* values <0.05 were considered significant.

Results

HIV-2 disease was associated with a marked depletion of memory B cells

We investigated here, for the first time, memory B-cell disturbances during HIV-2 infection. HIV-2+ patients showed no significant alterations in either the total numbers of peripheral blood B cells or the proportion of B cells within total PBMC as compared with seronegative individuals (Table 1). Memory B-cell populations were assessed by flow cytometry within PBMC, as illustrated in Figure 1.

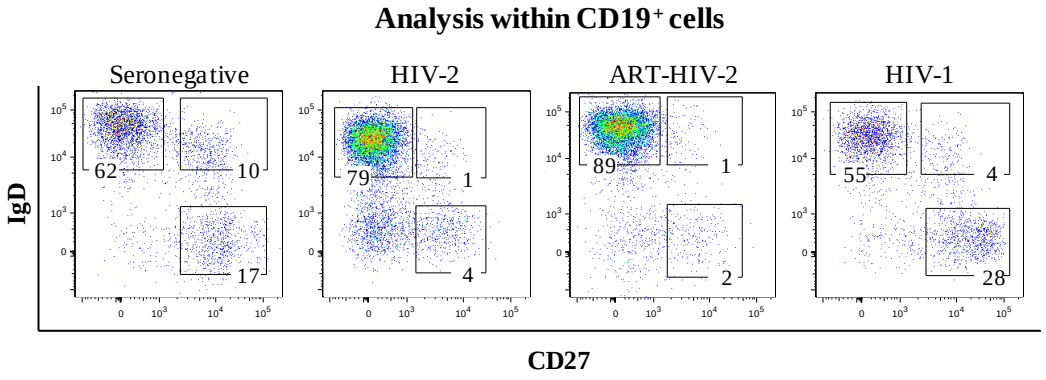

Figure 1. Flow-cytometric analysis of B-cell subsets. Representative dot-plots of the flow cytometric analysis of circulating B cells ($CD19^+CD3^-$), according to the surface expression of IgD and CD27. Age-matched individuals are shown. Data refer to one seronegative (31 years old, 778 $CD4^+$ T cells/µl), one untreated HIV-2+ (34 years old, 321 $CD4^+$ T cells/µl, undetectable viremia), one treated (ART) HIV-2+ (34 years old, 326 $CD4^+$ T cells/µl, undetectable viremia) and one untreated HIV-1+ (30 years old, 306 $CD4^+$ T cells/µl, 1814256 HIV-1 RNA copies/ml) individuals. Numbers inside quadrants represent the proportion of B cells expressing the respective markers.

A marked reduction in the proportion of memory B cells ($CD27^+$) was found in HIV-2+ individuals (Figure 1 and 2A), both in comparison with seronegative and HIV-1+

individuals with similar degrees of CD4$^+$ T-cell depletion (Table 1). Of note, numbers of circulating memory B cells were significantly lower in HIV-2 individuals than in seronegatives (Supplemental Figure 1).

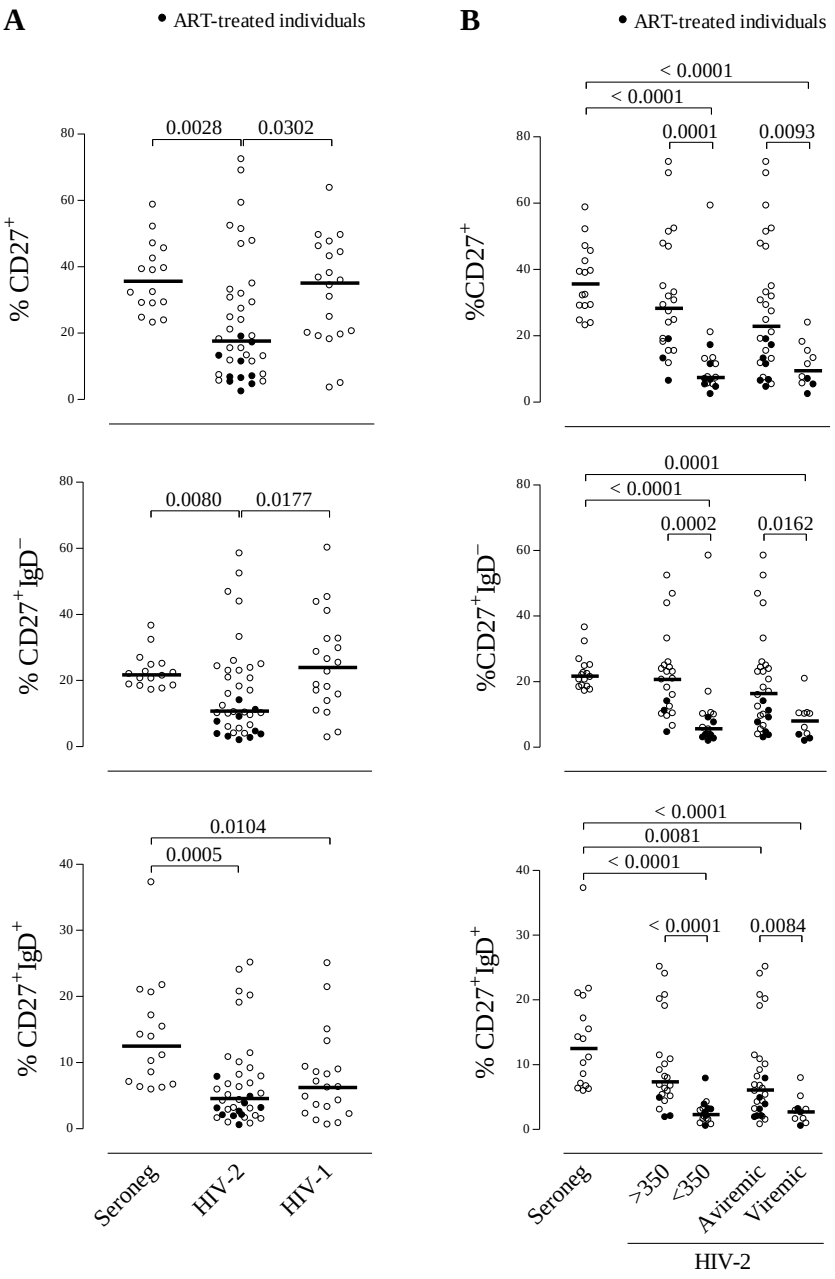

Figure 2. Memory B-cell subset frequency in HIV-2 infection. (A) Relative proportions of total memory cells (CD27$^+$, top graph), switched memory cells (CD27$^+$IgD$^-$, middle graph) and unswitched memory cells (CD27$^+$IgD$^+$, bottom graph), within total B cells, in the HIV-2 cohort, as well as seronegative (Seroneg) and HIV-1+ individuals. **(B)** HIV-2+ patients were further subdivided according to disease stage (early: >350 CD4$^+$ T cells/µl; late: <350 CD4$^+$ T cells/µl) and levels of plasma viral load (aviremic: undetectable plasma viral load; viremic: detectable). The frequencies of the B-cell subsets described in (A) were compared between seronegatives and all sub-groups of HIV-2+ individuals. Each dot represents one individual and bars indicate median. Filled circles refer to ART-treated individuals. Statistical analysis was performed using the Mann-Whitney test and the significant *P* values are shown.

Memory B-cell loss was strongly correlated with $CD4^+$ T-cell depletion and immune activation, assessed both in terms of T-cell activation and serum β2-microglobulin, in HIV-2+ individuals (Table 2 and Supplemental Table 2 for absolute B-cell counts). In agreement, this loss was significantly more marked in advanced as compared to early stage HIV-2 disease (less or more than 350 $CD4^+$ T cells; Figure 2B and Supplemental Figure 1B for absolute B-cell counts).

Table 2. *Relationship between B-cell subsets and markers of disease progression.*

		Analysis within B cells			
	% B cells	**%$CD27^-IgD^+$**	**%$CD27^+$**	**%$CD27^+IgD^+$**	**%$CD27^+IgD^-$**
HIV-2 (n=38)					
$CD4^+$ T cells/µl	-0.0222;0.8947	**-0.5551;0.0003**	**0.6001;<0.0001**	**0.7591;<0.0001**	**0.5717;0.0002**
Viremia, HIV RNA cp/ml	0.0167;0.9209	**0.3697;0.0223**	**-0.4214;0.0084**	**-0.4250;0.0078**	**-0.3981;0.0133**
%HLA-DR^+CD38^+ within CD4[a]	0.2132;0.1988	**0.4842;0.0021**	**-0.5541;0.0003**	**-0.7152;<0.0001**	**-0.5036;0.0013**
%HLA-DR^+CD38^+ within CD8[a]	0.1664;0.3180	**0.4323;0.0067**	**-0.4641;0.0033**	**-0.6157;<0.0001**	**-0.4224;0.0082**
β2-microglobulin (mg/l)[b]	-0.0327;0.8638	**0.5894;0.0006**	**-0.6416;0.0001**	**-0.7961;<0.0001**	**-0.5877;0.0006**
HIV-1 (n=20)					
$CD4^+$ T cells/µl	-0.0911;0.7026	-0.2295;0.3304	0.2528;0.2822	**0.6566;0.0017**	0.0391;0.8700
Viremia, HIV RNA cp/ml	0.0866;0.7165	-0.0934;0.6953	0.1574;0.5074	**-0.5467;0.0126**	0.3614;0.1174
%HLA-DR^+CD38^+ within CD4[a]	-0.0730;0.7598	0.1068;0.6539	-0.0760;0.7501	**-0.5709;0.0086**	0.1873;0.4291
%HLA-DR^+CD38^+ within CD8[a]	0.0008;0.9975	-0.2685;0.2523	0.2941;0.2082	-0.3820;0.0965	**0.5759;0.0079**
β2-microglobulin (mg/l)[b]	-0.4180;0.1369	-0.0550;0.8518	0.1342;0.6474	-0.3934;0.1640	0.2396;0.4094

Spearman's correlation coefficient was used and results are expressed as r;*P*, with significant correlations in bold.

[a]Frequency of cells co-expressing the activation markers HLA-DR and CD38 within $CD4^+$ and $CD8^+$ T cells (median, range) – HIV-2: 3.4%, 0.4-23.5% (CD4), and 15.1%, 0.6-69.5% (CD8); untreated HIV-1: 5.5%, 0.3-34.8% (CD4), and 23.7%, 1.4-62.2% (CD8). No significant differences were found between infected cohorts, which exhibited significantly higher levels than seronegative controls (CD4: 1.1%, 0.7-2.0%; CD8: 2.7%, 1.3-22.7%).

[b]β2-microglobulin serum levels were assessed for 30 HIV-2- (median: 2.6 mg/l, range: 1.1-7.8 mg/l) and 14 HIV-1- (median: 2.4 mg/l, range: 1.3-6.0 mg/l) infected individuals, with no statistically significant differences being observed between cohorts.

Additionally, an association was found between memory B-cell loss and viremia (Table 2 and Supplemental Table 2 for absolute B-cell counts). Accordingly, HIV-2+ patients with measurable viremia featured significantly less memory B cells than those with undetectable viremia (Figure 2B and Supplemental Figure 1B), despite the highest measured level being only 34314 RNA copies/ml (Table 1).

No such correlations were found in the untreated HIV-1 cohort (Table 2 and Supplemental Table 2 for absolute B-cell counts), despite the viremia being, on average, two-log higher than in the HIV-2 cohort (Table 1). The differences in memory B-cell loss between the two infections were particularly marked when groups of infected individuals in advanced disease stage or with detectable viremia were compared (Supplemental Figure 2A, and Supplemental Tables 3 and 4).

In spite of the distinct viremia, comparable amounts of cell-associated viral load (proviral DNA) were observed in the two infections (Table 1; Supplemental Tables 3 and 4), as previously reported [31-33]. Notably, no significant correlations were found between levels of proviral DNA and frequency of memory B cells in either infection (P>0.05).

HIV-1 has also been associated with an expansion of peripheral blood immature ($CD27^{-}CD10^{+}$) B cells that was shown to be directly correlated with circulating IL-7 levels, and been suggested to arise from lymphopenia-induced IL-7-mediated homeostasis [35]. We have previously shown that serum IL-7 is significantly increased in HIV-2 infection in strong association with $CD4^{+}$ T-cell levels [36]. In fact, we also observed a significant expansion of $CD27^{-}CD10^{+}$ B cells in HIV-2+ patients (median: 1.09%, range: 0.09-7.95; P=0.0007), as compared to seronegatives (median: 0.39%, range: 0.10-1.41). Nevertheless, we found no significant correlations between the frequency of $CD27^{-}CD10^{+}$ B cells with serum IL-7 levels in the HIV-2 cohort (n=35; serum IL-7 levels median: 12.21 pg/ml, range: 2.26-28.70; r=0.1859, P=0.2850), suggesting that other mechanisms are modulating the expansion of immature B cells during HIV-2 infection.

Although, the HIV-2 cohort included 10 individuals under ART (Supplemental Table 1), the B-cell imbalances were also found when only untreated subjects were compared to seronegatives (Supplemental Figure 3A).

Moreover, ART-treated patients showed a significantly lower frequency of memory B cells than their untreated counterparts (Supplemental Figure 3A), which may reflect their prolonged infection, as estimated below, and the poor immunological recovery under ART typically observed in HIV-2 infection (Supplemental Table 1).

Overall, HIV-2 infection was associated with progressive loss of memory B cells.

Both switched and unswitched memory B cells were lost during HIV-2 disease

The $CD27^+$ B-cell subset can be further subdivided in terms of class-switched and unswitched immunoglobulin production, assessed here by IgD surface expression [37]. Class-switched memory B cells ($CD27^+IgD^-$) are mainly generated in germinal centres and play a key role in adaptive immune responses, whilst unswitched $CD27^+IgD^+$ B cells are thought to include largely marginal zone B cells, and are known to play a role in protection against encapsulated bacteria [37].

Memory B-cell loss observed during HIV-2 disease was related to a pronounced depletion of cells with a class-switched phenotype (Figure 1 and 2A; Supplemental Figure 1 for absolute counts), which showed strong positive correlations with markers of disease progression (Table 2 and Supplemental Table 2 for absolute B-cell counts). No such correlations were observed in the HIV-1 cohort (Table 2 and Supplemental Table 2), and the switched memory B-cell depletion was much lower in HIV-1 than in HIV-2 infection (Figure 1 and 2A; Supplemental Figure 1 for absolute counts). Moreover, in the HIV-2 cohort, switched memory B-cell frequency was significantly lower in patients with less than 350 $CD4^+$ T cells/µl, as compared to early stage disease, as well as in patients with detectable viremia *versus* their aviremic counterparts (Figure 2B; Supplemental Figure 1B for absolute counts). This was also

observed for untreated and ART-treated HIV-2+ individuals, when analysed separately (Supplemental Figure 3B), but was not documented for HIV-1 infection (Supplemental Figure 2B). Notably, despite both infections exhibiting similar levels of immune activation (Table 2), as previously reported [12], the impairment in switched memory B-cell preservation seemed to be more strongly linked to the persistent immune activation in HIV-2 than in HIV-1 infection (Table 2 and Supplemental Table 2 for absolute B-cell counts).

Conversely, the loss of unswitched (IgD^+) memory B cells in HIV-2 infection was comparable to that observed in HIV-1 infection (Figure 2A and Supplemental Figure 1A for absolute counts). This loss was positively correlated with markers of disease progression in both HIV-1 and HIV-2 cohorts (Table 2 and Supplemental Table 2 for absolute B-cell counts), being significantly more pronounced in patients with $CD4^+$ T-cell counts below 350 cells/µl or detectable viremia (Figure 2B; Supplemental Figures 1B and 2C).

Of note, both switched and unswitched memory B cells were markedly decreased in HIV-2+ patients under ART (Supplemental Figure 3B and 3C, respectively), which may be, at least in part, related to the more prolonged length of disease. Given the slow rate of $CD4^+$ T-cell decline, it is expected that HIV-2+ patients have been infected for much longer than untreated HIV-1+ individuals. Our estimation of the length of follow-up, for those patients for whom these data were available, was in agreement with this possibility (median months for treated HIV-2: 97, range: 8-242, n=10; untreated HIV-2: 71, range: 24-235, n=16; untreated HIV-1: 75, range: 13-184, n=9).

Notably, B-cell imbalances did not correlate with age in all the cohorts ($p>0.05$).

Altogether, progressive memory B-cell loss during HIV-2 infection was due to a depletion of both switched and unswitched memory B cells.

Memory B-cell imbalances did not translate into decreased immunoglobulin levels

Notwithstanding the generalized memory B-cell loss, most HIV-2+ patients showed increased levels of IgG, as previously reported [22, 23], whilst maintaining IgM and IgA titers within the normal range (Supplemental Figure 4A).

Additionally, we quantified serum levels of antibodies against HIV-2 *env* glycoproteins gp36 and gp125 (C2-C3 region) as previously described [29]. No significant differences were observed in specific antibody levels when HIV-2+ patients were sub-divided, either according to $CD4^+$ T-cell counts, or viremia (P>0.05). Nevertheless, whilst IgG, IgM and IgA levels did not significantly differ between untreated and treated HIV-2 infection, HIV-2+ individuals under ART had significantly lower levels of specific antibodies against the C2-C3 region of gp125 (Supplemental Figure 4B).

Overall, HIV-2+ patients did not feature major impairments in total immunoglobulin levels or HIV-2-specific antibody production, despite the observed memory B-cell imbalances.

BAFF levels increased during HIV-2 disease, particularly in the presence of detectable viremia, in direct association with memory B-cell disturbances

B-cell survival and differentiation rely on BAFF, a cytokine mainly produced by stromal cells, monocytes and T cells, detectable in the serum of healthy subjects [38-40]. BAFF levels were significantly higher in the HIV-2 cohort, as compared with seronegatives (Figure 3A). Notably, a direct correlation was found between serum BAFF levels and viremia in the HIV-2 cohort (r=0.4939, P=0.0195, n=12), and in agreement, significantly higher BAFF titres were observed in HIV-2+ patients with detectable viremia, both in relation to aviremic individuals and seronegatives (Figure 3B, Supplemental Figure 5A); this was observed in untreated as well as in ART-treated HIV-2 groups (Supplemental Figure 5B). A similar relationship between serum BAFF

levels and viremia was observed in the HIV-1 cohort (r=0.5916, *P*=0.0428, n=12; and Supplemental Figure 5A).

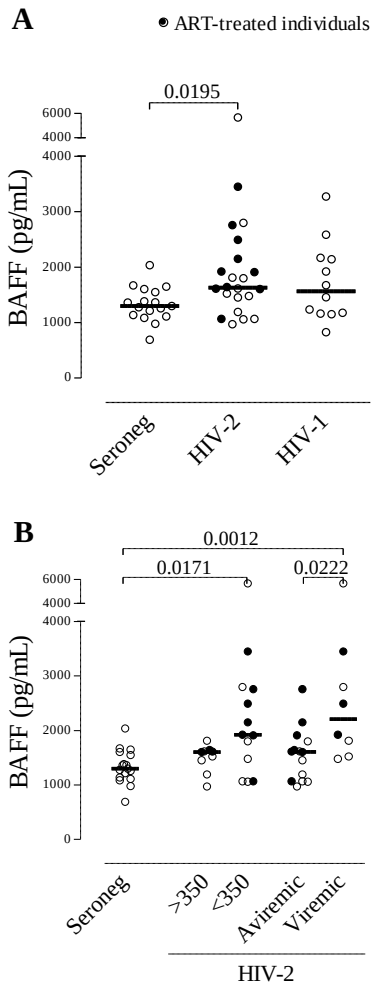

Figure 3. Serum BAFF levels in HIV-2 infection. (A) Serum BAFF levels in HIV-2+, HIV-1+, and seronegative (Seroneg) individuals. Due to sample availability, a distinct seronegative cohort was used (11 females/6 males; 39 (26-61) median years of age; 63.85 (40.10-76.90) median $CD4^+$ T-cell frequency; 908 (537-1312) median $CD4^+$ T cells/µl; 8.15 (2.56-18.19) median B-cell frequency; 163 (63-383) median B cells/µl), as well as representative subgroups of the infected cohorts (HIV-2, including the 10 treated subjects: 13 females/9 males; 56 (19-72) median years of age; 28.02 (4.51-63.20) median $CD4^+$ T-cell frequency; 337 (52-1336) median $CD4^+$ T cells/µl; 200 (200-34314) median RNA copies/ml; 108 (5-1002) median proviral DNA copies/10^6 PBMC; 7.41 (2.89-19.70) median B-cell frequency; 132 (30-369) median B cells/µl; and untreated HIV-1: 3 females/9 males; 42 (23-61) median years of age; 32.85 (7.71-74.4) median $CD4^+$ T-cell frequency; 431 (77-1425) median $CD4^+$ T cells/µl; $1.4x10^4$ (40-$4.5x10^6$) RNA copies/ml; 88 (5-573) median proviral DNA copies/10^6 PBMC; 6.64 (2.08-15.60) median B-cell frequency; 112 (56-324) median B cells/µl). **(B)** The HIV-2 cohort was further stratified according to disease stage (early: >350 $CD4^+$ T cells/µl; late: <350 $CD4^+$ T cells/µl) and levels of plasma viral load (aviremic: undetectable; viremic: detectable), and the BAFF levels were compared between seronegatives and all groups of HIV-2+ individuals. Each dot represents one individual and bars indicate median. Filled circles refer to ART-treated individuals. Statistical analysis was performed using the Mann-Whitney test and the significant *P* values are shown.

Although no significant correlations were found between serum BAFF levels and the degree of $CD4^+$ T-cell depletion, advanced HIV-2 disease (less than 350 $CD4^+$ T cells/µl) was associated with higher BAFF levels (Figure 3B). Moreover, HIV-2+ individuals under ART showed a significant increase in BAFF as compared to seronegatives irrespective of viremia possibly related to their reduced $CD4^+$ T-cell counts (Supplemental Figure 5B).

Of note, HIV-2+ patients showed significant inverse correlations between serum BAFF levels and frequency of total (r=-0.5456, *P*=0.0086, n=22) and switched memory B cells (r=-0.5247, *P*=0.0122, n=22). Such correlations were not found in the HIV-1 cohort (*P*>0.05).

In conclusion, HIV-2 infection was associated with an increase in serum BAFF levels that correlated with memory B-cell loss.

Discussion

We report here, for the first time, that despite its relatively "benign" course and low viremia, HIV-2 disease progression was associated with major losses of both switched and unswitched memory B cells and elevated serum BAFF levels. Additionally, we found that these disturbances were not recovered by ART.

We showed that memory B-cell levels negatively correlated with T-cell activation in HIV-2+ individuals, suggesting a close association between B-cell disturbances and hyper-immune activation, even in the context of low viremia. This is in agreement with previous reports associating B-cell dysregulation during HIV-1 disease with hyper-immune activation [3, 4].

In order to account for the possible impact of $CD4^+$ T-cell lymphopenia on B-cell disturbances, we compared HIV-1+ and HIV-2+ individuals with similar degrees of $CD4^+$ T-cell depletion, despite differences in length of disease and viremia. Importantly, memory B-cell loss, particularly of the switched memory pool, was much

more marked in HIV-2 than HIV-1 infection, despite the clearly better prognosis and the much lower amounts of circulating virus in HIV-2 infection.

A recent report associated the loss of switched memory B cells in HIV-1 infection with impairment in the ability of B cells to respond to IL-2 or other γc cytokines, leading to an increase in their susceptibility to apoptosis [4]. Although this possibility was not excluded in the current study of HIV-2+ patients, our previous data support a relatively better preserved ability to produce and respond to γc cytokines in HIV-2, as compared to HIV-1 infection [36, 41, 42].

The marked levels of switched memory B-cell depletion in HIV-2 infection were unlikely to be solely attributable to loss of the small subset of IgM-only ($CD27^{+}IgD^{-}IgM^{+}$) memory B cells [43, 44]. Nevertheless, it would be of interest to address in future studies the detailed phenotype of memory B cells, including levels of expression of IgM and CD21, whose loss has been associated with ongoing HIV replication and disease progression in HIV-1+ individuals [37].

It is also possible that cell redistribution contributed, at least in part, to the reduction of circulating memory B cells, emphasizing the relevance of tissue studies. Moreover, given the expected prolonged length of HIV-2 disease, it is plausible that the marked loss of switched memory B cells may be related to cumulative lymphoid tissue damage, and consequent disruption of the generation of germinal centres where these cells are produced, and/or of the microenvironment required for their survival, ultimately reaching an irreversible level. Accordingly, a more marked switched memory B-cell loss was found in HIV-2+ patients under long-term antiretroviral treatment, who were estimated to have been infected for a longer period of time.

To our knowledge, there are no data on HIV-2-infected secondary lymphoid organs (SLO). The persistent immune activation observed during HIV-2 infection [12] may be associated with SLO inflammation and collagen deposition with progressive disruption of their architecture, as has been described during HIV-1 infection [45]. HIV-1 has been shown to mainly reside in lymphoid tissues, trapped in the follicular

dendritic cell (FDC) network, potentially contributing to progressive disruption of germinal centre responses and limiting the survival signals required for memory B-cell maintenance [46, 47]. It is also possible that low levels of HIV-2 replication occur in SLO, contributing to the hyper-activated immune state and the putative progressive tissue damage. In fact, the cytopathicity documented upon HIV-2 infection of human lymphoid tissue explant cultures was shown to be comparable to that observed for HIV-1 [48].

Importantly, we and others have reported similar levels of cell-associated viral burden in HIV-1+ and HIV-2+ patients, as assessed by the levels of proviral DNA within PBMC [31-33]. Thus, the ability to disseminate and establish a reservoir of infected cells is apparently similar in both infections. Furthermore, we have recently provided evidence of a significant degree of ongoing viral replication in HIV-2+ individuals based on the levels of viral *gag* and *tat* gene transcripts within PBMC [31]. These data raised questions about the control of HIV-2 latency. It is plausible that HIV-2 continuously replicates in SLO at low levels, possibly by cell-to-cell mediated transmission. In spite of this putative HIV-2 replication being locally contained, given the reduced viremia, it may have a long-term impact, contributing to accelerated immune-senescence. Additionally, it may contribute to the poor immunological recovery that is usually observed in HIV-2+ patients receiving ART [20, 31, 49, 50], highlighting the importance of rethinking the guidelines on when to start ART in HIV-infected patients [51].

Our findings are particularly relevant in view of the cumulative data suggesting that HIV-1+ patients under apparently effective ART have significant degrees of replication in SLO [52, 53]. These low levels of viral replication were suggested to contribute to a pro-inflammatory state and to the related complications found in long-term treated HIV-1+ patients [53].

Notably, in spite of the reduced amounts of plasma HIV-2 load, irrespective of disease stage, we found that the presence of detectable viremia was associated with significantly lower levels of memory B cells. These results suggest a direct role of the free virions in B-cell disturbances. Alternatively, the presence of detectable virus may

itself represent indirect evidence for the disruption of the FDC network, which eventually becomes unable to contain the produced virions.

BAFF has been shown to be critical for B-cell maturation and survival upon bone-marrow egress [38]. Of note, we found that serum BAFF levels were increased in HIV-2-infected patients, particularly in association with viremia, in agreement with previous reports in HIV-1 infection [39, 40]. Moreover, HIV-2+ individuals under ART, who had longer follow-up, featured significant increases in BAFF levels irrespective of viremia, which may contribute to their heightened inflammatory state and B-cell imbalances, as has been suggested in HIV-1 infection [8, 39, 40, 54].

Overall, we showed, for the first time, that despite the reduced amount of circulating virus, HIV-2 infection is associated with a marked depletion of both switched and unswitched memory B cells, in association with an increase in serum BAFF levels. Our data provide evidence that prolonged HIV disease, even in the absence of detectable viremia, leads to an irreversible damage of memory B-cell homeostasis.

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Supplemental Data

Supplemental Table 1. *Clinical and epidemiological characteristics of the therapy naïve and treated HIV-2-infected individuals.*

	Seronegatives	Untreated HIV-2	ART-HIV-2[a]
Number [male/female]	16 [6/10]	28 [9/19]	10 [5/5]
Age, years	44 (27-57)	54 (19-78)	57 (34-62)**
Caucasian/black	15/1	15/13	6/4
CD4$^+$ T cells, %	59 (40-77)	40 (10-66)**	26 (4-40)$^{***/\#\#}$
CD4$^+$ T cells/µl	818 (518-1312)	553 (52-1511)*	288 (84-554)$^{***/\#\#}$
Viremia, HIV RNA cp/ml	-	200 (200-26263)	200 (200-34314)
Proviral DNA, cp/10^6 PBMC	-	5 (5-1033)	130 (5-726)
B cells, %	6 (3-13)	6 (3-20)	10 (4-17)*
B cells/µl	120 (63-369)	131 (30-369)	135 (63-313)

Data are expressed as median, with limits in brackets. Statistical analysis was performed with Mann-Whitney tests. $^{*}P< 0.05$; $^{**}P< 0.01$; $^{***}P< 0.001$ in comparison with seronegatives.
$^{\#\#}P< 0.01$ for comparisons between infected cohorts.
[a]ART regimens were as follows (number of ART-HIV-2 patients): 3TC+AZT+LPV/r (3); 3TC+d4T+SQV/r (2); 3TC/AZT+IDV/r (1); 3TC+AZT+IDV (1); ABC+3TC+AZT (3). Nucleoside analog reverse transcriptase inhibitors (NRTIs): AZT – Zidovudine, 3TC- Lamivudine, ABC- abacavir, d4T- Stavudine, r- ribavirine. Protease inhibitors (PIs)- LPV- Lopinavir, IDV- Indinavir, SQV- Saquinavir.

Supplemental Table 2. *Relationship between absolute counts of circulating B-cell populations and markers of disease progression in the HIV-2 and HIV-1 cohorts.*

		B cells/µl			
	Total B cells/µl	**$CD27^{-}IgD^{+}$**	**$CD27^{+}$**	**$CD27^{+}IgD^{+}$**	**$CD27^{+}IgD^{-}$**
HIV-2 (n=38)					
$CD4^{+}$ T cells/µl	**0.3522;0.0301**	0.1322;0.4288	**0.7561;<0.0001**	**0.7830;<0.0001**	**0.7137;<0.0001**
Viremia, HIV RNA cp/ml	-0.0754;0.6526	0.0557;0.7396	**-0.4280;0.0074**	**-0.3842;0.0172**	**-0.4273;0.0074**
%HLA-$DR^{+}CD38^{+}$ within CD4[a]	0.0153;0.9275	0.1899;0.2535	**-0.5002;0.0014**	**-0.5512;0.0003**	**-0.4611;0.0036**
%HLA-$DR^{+}CD38^{+}$ within CD8[a]	-0.0390;0.8164	0.1324;0.4281	**-0.4466;0.0049**	**-0.4964;0.0015**	**-0.4166;0.0093**
β2-microglobulin (mg/l)[b]	-0.2110;0.2631	-0.0039;0.9837	**-0.6758;<0.0001**	**-0.7367;<0.0001**	**-0.6326;0.0002**
HIV-1 (n=20)					
$CD4^{+}$ T cells/µl	0.2144;0.3639	0.2023;0.3923	0.2866;0.2206	**0.5957;0.0056**	0.2153;0.3620
Viremia, HIV RNA cp/ml	0.0098;0.9673	-0.1340;0.5732	0.0919;0.7001	-0.4339;0.0559	0.2027;0.3914
%HLA-$DR^{+}CD38^{+}$ within CD4[a]	-0.2506;0.2866	-0.3106;0.1825	-0.1971;0.4050	-0.5794;0.0074	-0.0941;0.6932
%HLA-$DR^{+}CD38^{+}$ within CD8[a]	-0.0669;0.7792	-0.2857;0.2220	0.1985;0.4015	-0.3350;0.1488	0.3168;0.1736
β2-microglobulin (mg/l)[b]	-0.3740;0.1877	-0.4462;0.1098	-0.1209;0.6806	**-0.5433;0.0447**	-0.0264;0.9286

Spearman's correlation coefficient was used and results are expressed as R;*P*, with significant correlations in bold.

[a]Frequency of cells co-expressing the activation markers HLA-DR and CD38 within $CD4^{+}$ and $CD8^{+}$ T cells (median, range) – HIV-2: 3.4%, 0.4-23.5% (CD4), and 15.1%, 0.6-69.5% (CD8); untreated HIV-1: 5.5%, 0.3-34.8% (CD4), and 23.7%, 1.4-62.2% (CD8). No significant differences were found between infected cohorts, which exhibited significantly higher levels than seronegative controls (CD4: 1.1%, 0.7-2.0%; CD8: 2.7%, 1.3-22.7%).

[b]β2-microglobulin serum levels were assessed for 30 HIV-2 (median: 2.6 mg/l, range: 1.1-7.8 mg/l) and 14 HIV-1 (median: 2.4 mg/l, range: 1.3-6.0 mg/l) infected individuals, with no statistically significant differences being observed between cohorts.

Supplemental Table 3. *Clinical and epidemiological characteristics of the HIV-2 and HIV-1 cohorts stratified according to degree of CD4$^+$ T-cell depletion.*

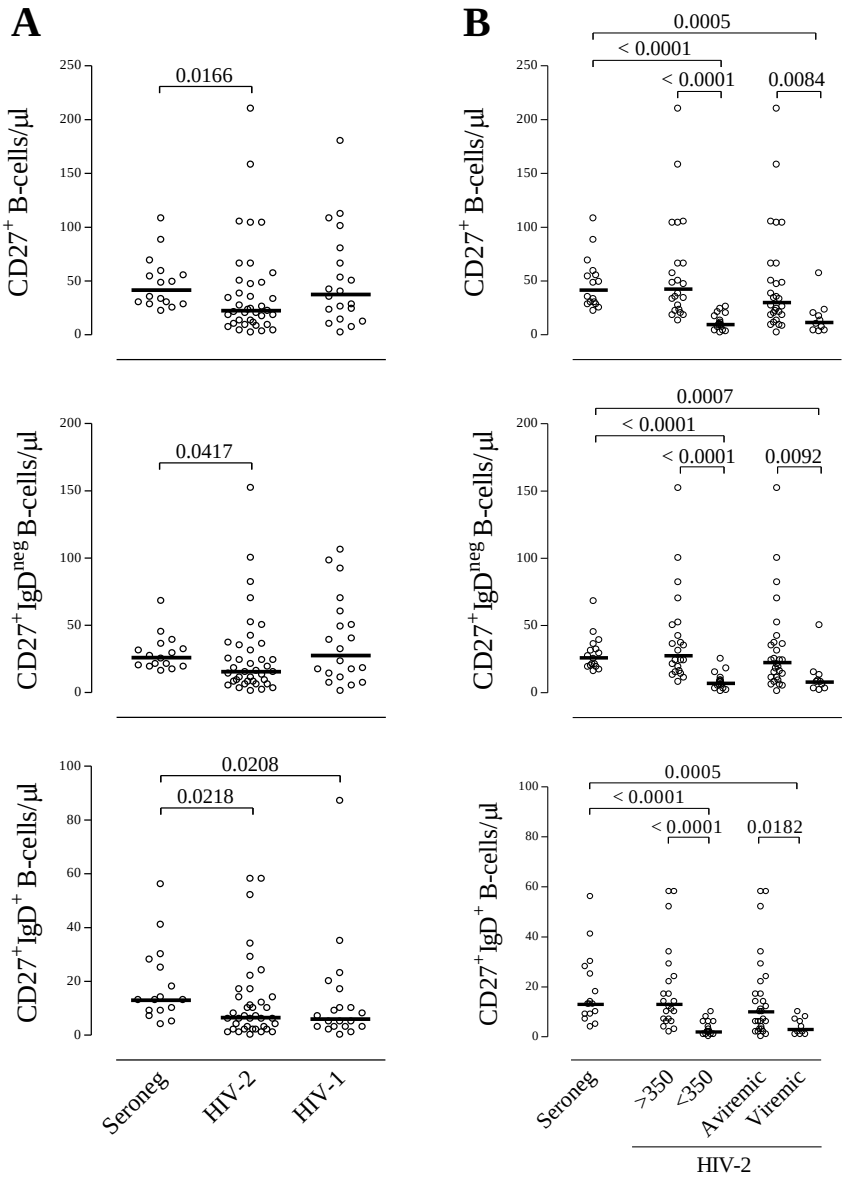
	Seroneg	HIV-2		HIV-1	
		>350 CD4$^+$/µl	**<350** CD4$^+$/µl	**>350** CD4$^+$/µl	**<350** CD4$^+$/µl
Number [male/female]	16 [6/10]	22 [7/15]	16 [7/9]	10 [7/3]	10 [8/2]
Age, years	44 (27-57)	56 (28-78)*	51 (19-63)	41 (27-61)	35 (23-49)$^{\#}$
Caucasian/black	15/1	13/9	8/8	10/0	5/5
CD4$^+$ T cells, %	59 (40-77)	45 (22-66)$^{**/+++}$	21 (4-32)***	46 (17-74)*	14 (2-32)$^{***/+++}$
CD4$^+$ T cells/µl	818 (518-1312)	661 (406-1511)$^{+++}$	179 (52-344)***	885 (372-1848)	298 (18-344)$^{***/+++}$
Viremia (HIV RNA cp/ml)	-	200 (200-13627)	200 (200-34314)	$5x10^3$ ($40-19x10^3$)	$5x10^5$ ($71-4470x10^3$)$^{++/\#\#}$
Proviral DNA (cp/10^6 PBMC)	-	78 (5-1033)	57 (5-1002)	81 (5-573)	41 (5-975)
B cells, %	6 (3-13)	7 (3-17)	6 (3-20)	5 (2-11)	6 (2-16)
B cells/µl	120 (63-369)	141 (52-357)	100 (30-369)	118 (56-406)	117 (33-267)

Data are expressed as median, with limits in brackets. Statistical analysis was performed with Mann-Whitney tests. Seroneg: seronegatives.
$^{*}P< 0.05$; $^{**}P< 0.01$; $^{***}P< 0.001$ in comparison with seronegatives. $^{++}P< 0.01$; $^{+++}P< 0.001$ in comparison with the reciprocal subgroup (>350 *versus* <350). $^{\#}P< 0.05$; $^{\#\#}P< 0.01$ for comparisons between infected cohorts.

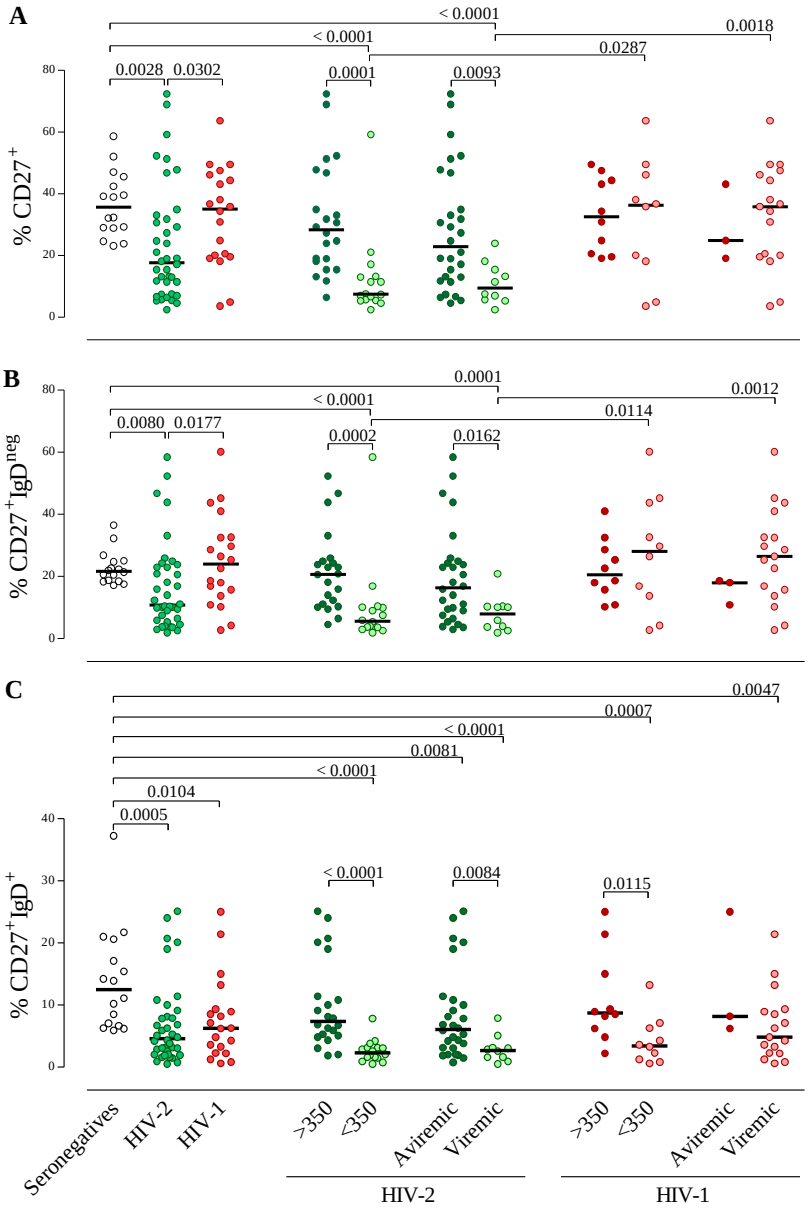
Supplemental Table 4. *Clinical and epidemiological characteristics of the HIV-2 and HIV-1 cohorts stratified according to viremia status.*

	Seroneg	HIV-2		HIV-1	
		Aviremic	Viremic	Aviremic	Viremic
Number [male/female]	16 [6/10]	28 [9/19]	10 [5/5]	3 [2/1]	17 [13/4]
Age, years	44 (27-57)	56 (28-78)	52 (19-62)	47 (30-61)	38 (23-59)
Caucasian/black	15/1	17/11	4/6	3/0	12/5
CD4$^+$ T cells, %	59 (40-77)	37 (13-66)$^{***/+}$	24 (4-53)***	60 (59-74)$^{\#}$	27 (2-56)$^{***/++}$
CD4$^+$ T cells/µl	818 (518-1312)	546 (52-1511)$^{*/+}$	249 (84-596)***	965 (521-1425)	341 (18-1848)**
Viremia (HIV RNA cp/ml)	-	<200	$7.6x10^3$ (742-34314)	<40	$1.9x10^4$ ($71-4470x10^3$)
Proviral DNA (cp/10^6 PBMC)	-	5 (5-1033)$^{+}$	187 (5-1002)	104 (5-324)	53 (5-975)
B cells, %	6 (3-13)	7 (3-17)	7 (3-20)	10 (4-11)	6 (2-16)
B cells/µl	120 (63-369)	135 (37-357)	131 (30-369)	125 (92-324)	111 (33-406)

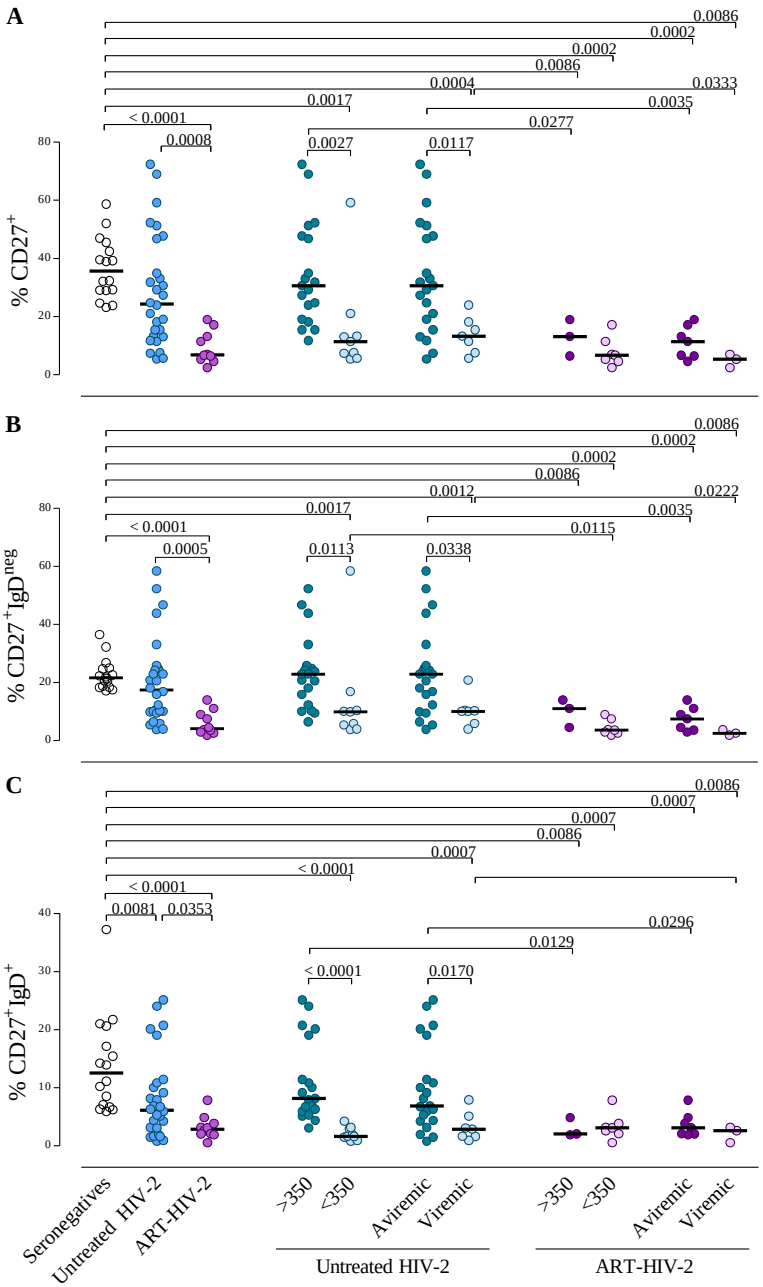
Data are expressed as median, with limits in brackets. Statistical analysis was performed with Mann-Whitney tests. Seroneg: seronegatives.
$^{*}P< 0.05$; $^{**}P< 0.01$; $^{***}P< 0.001$ in comparison with seronegatives. $^{+}P< 0.05$; $^{++}P< 0.01$ in comparison with the reciprocal subgroup (Aviremic *versus* Viremic). $^{\#}P< 0.05$ for comparisons between infected cohorts.



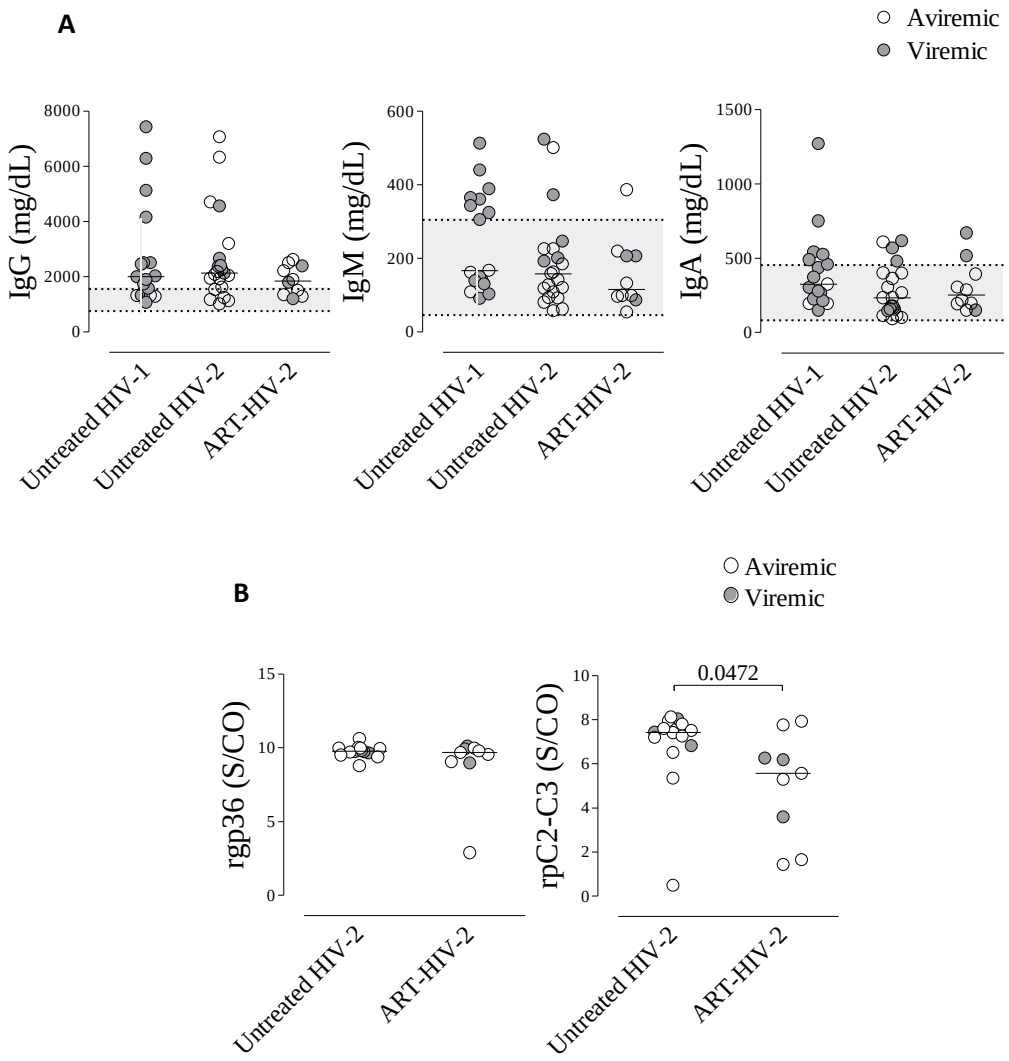
Supplemental Figure 1. Absolute numbers of memory B-cell subsets in HIV-2-infected individuals. (**A**) Circulating numbers of total memory cells ($CD27^+$), switched memory cells ($CD27^+IgD^{neg}$) and unswitched memory cells ($CD27^+IgD^+$) in HIV-2 infected, as well as seronegative (Seroneg) and HIV-1+ individuals. **(B)** HIV-2+ patients were further subdivided according to disease stage (early: >350 $CD4^+$ T cells/µl; late: <350 $CD4^+$ T cells/µl) and levels of plasma viral load (aviremic: undetectable plasma viral load; viremic: detectable) and the absolute counts of memory B-cell populations are shown. Each dot represents one individual and bars indicate median. Statistical analysis was performed using the Mann-Whitney test and significant *P* values are shown.



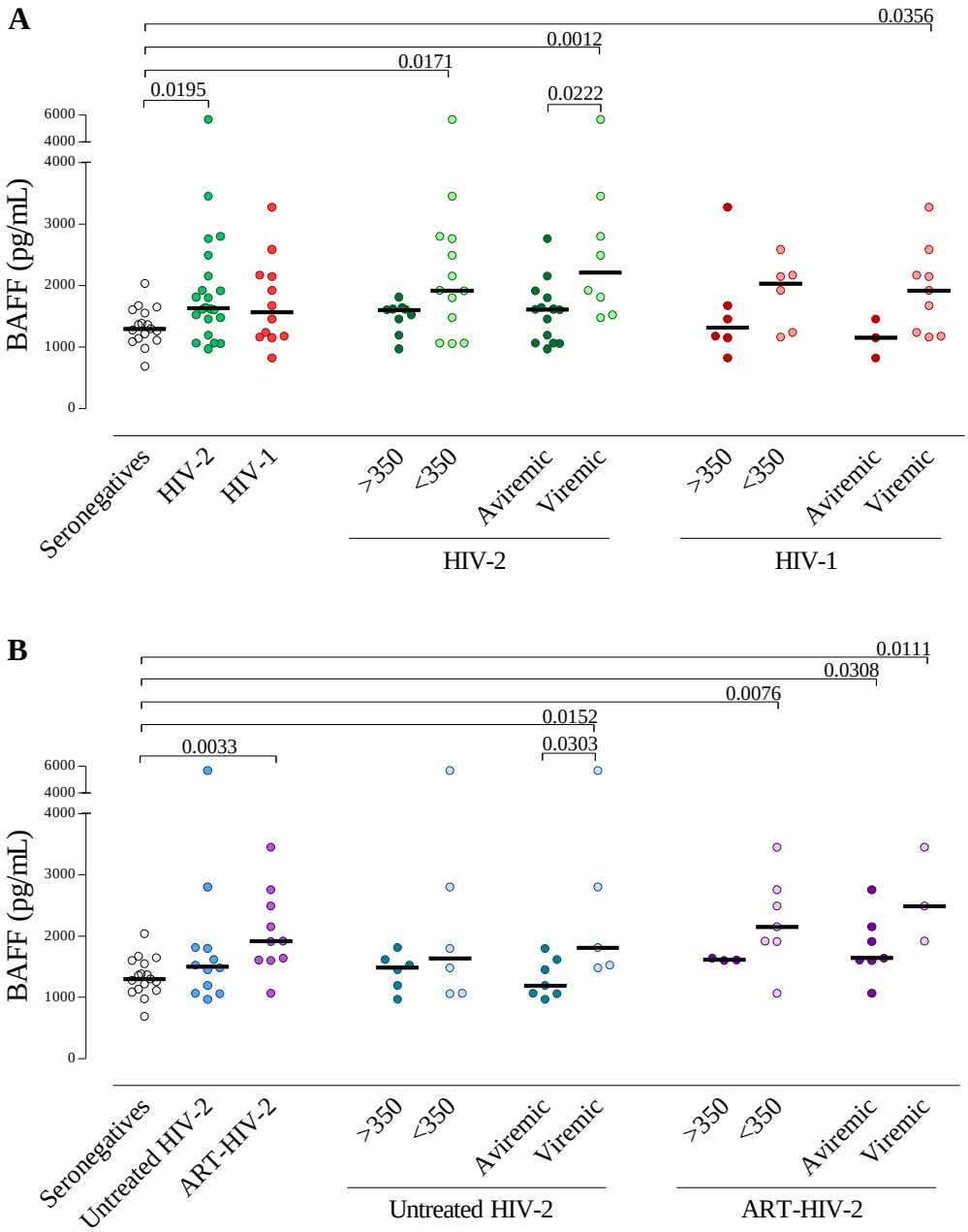
Supplemental Figure 2. B-cell disturbances in HIV-2 as compared to HIV-1 groups of infected patients stratified to disease stage and viremia status. Patients were divided according to disease stage (early: >350 $CD4^+$ T cells/µl; late: <350 $CD4^+$ T cells/µl) and levels of plasma viral load (aviremic: undetectable plasma viral load; viremic: detectable). Relative frequencies of (**A**) total memory cells ($CD27^+$), (**B**) switched memory cells ($CD27^+IgD^{neg}$) and (**C**) unswitched memory cells ($CD27^+IgD^+$) within total B cells. Comparisons were made between seronegatives and all groups of infected individuals. Each dot represents one individual and bars indicate median. Statistical analysis was performed using the Mann-Whitney test and significant *P* values are shown.



Supplemental Figure 3. B-cell disturbances in treated as compared to untreated HIV-2-infected patients stratified to disease stage and viremia status. Patients were divided according to disease stage (early: >350 $CD4^+$ T cells/µl; late: <350 $CD4^+$ T cells/µl) and levels of plasma viral load (aviremic: undetectable plasma viral load; viremic: detectable). Relative frequencies of (**A**) total memory cells ($CD27^+$), (**B**) switched memory cells ($CD27^+IgD^{neg}$) and (**C**) unswitched memory cells ($CD27^+IgD^+$) within total B cells. Comparisons were made between seronegatives and all groups of infected individuals. Each dot represents one individual and bars indicate median. Statistical analysis was performed using the Mann-Whitney test and significant *P* values are shown.



Supplemental Figure 4. Serum levels of immunoglobulin and of specific antibodies against HIV-2 envelope glycoproteins gp125 and gp36. (**A**) Serum levels of total IgG, total IgM, and total IgA in untreated HIV+ and in ART-treated HIV-2+ patients. (**B**) Serum levels of specific antibodies against gp36 and gp125 expressed as $OD_{clinical\ sample}/OD_{cut\text{-}off}$ (S/CO) ratios in HIV-2+ individuals. Each dot represents one individual. Patients with viremia below cut-off are shown as open dots and viremic patients as filled dots. Normal range of immunoglobulin values is represented by grey shading. Statistical analysis was performed using the Mann-Whitney test and significant *P* values are shown. Bars represent median.



Supplemental Figure 5. Serum BAFF levels in HIV-infected individuals stratified according to disease stage and viremia status. Patients were divided according to disease stage (early: >350 $CD4^+$ T cells/µl; late: <350 $CD4^+$ T cells/µl) and levels of plasma viral load (aviremic: undetectable plasma viral load; viremic: detectable). Comparisons were made between seronegatives and (A) groups of HIV-2 and HIV-1 infected individuals; (B) groups of untreated and treated HIV-2 infected individuals. Each dot represents one individual and bars indicate median. Statistical analysis was performed using the Mann-Whitney test and significant *P* values are shown.

CHAPTER 4:
CONCLUSIONS AND FUTURE PERSPECTIVES

Clarification of the immune responses underlying the more “benign” disease course that characterizes HIV-2 infection is of utmost importance for the general understanding of HIV/AIDS immunopathology. Furthermore, it may contribute to the identification of new potential targets to be used in ART-complementary immune-based therapy.

Chronic immune activation has long been recognized as a hallmark of HIV infection, constituting a stronger predictor of disease progression and concomitant $CD4^+$ T-cell depletion than viremia [1, 2]. Hence, strategies aimed at limiting immune activation may be beneficial to control the infection, possibly contributing to slow down disease progression.

Cells of the innate immune system constitute the primary line of defense against infection [3]. In acute HIV disease, innate immunity plays a major role in limiting viral replication and promoting the development of adaptive immune responses, whilst during chronic infection it may act as a modulator of immune activation [3].

We have previously shown that, despite the undetectable to low viremia found in HIV-2+ individuals, a comparable marked reduction in circulating pDC levels, associated with both $CD4^+$ T-cell depletion and T-cell activation, was observed in untreated HIV-1 and HIV-2 infections [4]. Moreover, the same over-expression of CD86 and PD-L1 molecules on circulating pDC was found in both infections, irrespective of disease stage and viremia [4]. Nevertheless, although, on a *per* cell basis, IFN-α production upon *in vitro* stimulation was observed to be equally impaired in viremic HIV-1+ and HIV-2+ patients, aviremic HIV-2+ individuals showed *in vitro* levels of IFN-α production similar to seronegatives [4].

These results suggested that mechanisms other than the direct infection of pDC could determine their depletion and activation during HIV-2 disease. Additionally,

these findings were in agreement with a lower *in vivo* IFN-α activity throughout the course of HIV-2 disease [4], possibly contributing to the slower heightening of immune activation and consequent lower rate of $CD4^+$ T-cell depletion observed in this infection [5].

In chapter 3.1., we sought to further address the role of innate immunity during HIV disease. An increased activation of both monocytes and mDC, with concomitant particularly marked PD-L1 up-regulation, also constituted a hallmark of HIV-2 infection. A pronounced loss of circulating mDC was observed in HIV infection, with no alterations in total monocyte numbers being found, despite the substantial expansion of a specific subset featuring the highest expression of co-stimulatory and inhibitory molecules (HLA-DR and PD-L1, respectively). Nevertheless, both monocytes and mDC from HIV-infected patients displayed a hyper-activated state that, in the context of HIV-2 infection, was accompanied by a more marked and significant up-regulation of PD-L1 expression. It is important to note that this occurred even in the absence of viremia and, more importantly, in early disease stages. These findings suggest that, in HIV-2+ individuals, the PD-1/PD-L1 inhibitory pathway could play a role in limiting immune activation from the early stages of infection. In agreement with this hypothesis, no major disturbances in the circulating levels of monocyte-produced cytokines and chemokines were found in the HIV-2 cohort, which typically displayed similar or lower titers of circulating TNF-α, IL-6, IL-10, MIP-1β and MCP-1 than those observed in HIV-1 infection. Moreover, monocyte activation and cytokine production ability, as well as mDC disturbances, appeared to be dissociated from plasma LPS levels in the context of HIV-2 disease, arguing against a major role for microbial translocation in HIV-2 immunopathology, as reported for several African populations [6, 7].

Taken together, our data suggest that the marked PD-L1 up-regulation observed on pDC, mDC and monocytes during HIV-2 disease may contribute to its slow disease course. The process underlying the up-regulation of this molecule is apparently independent, to a large degree, of both IFN-α and TLR-4-mediated stimulation. Hence, other mechanisms, possibly involving T-cell-dependent monocyte

activation and/or response to γc cytokines, may contribute to a better maintained balance between activating and inhibiting pathways in the context of HIV-2 infection, and warrants further investigation.

T-cell hyper-activation is strongly associated with HIV immunopathogenesis and progression to AIDS [1]. Nevertheless, as well as the expected increase in the surface expression of markers associated with stimulation, activated T cells have also been shown to up-regulate the expression of both PD-1 and PD-L1 expression [8], further supporting the possibility that inhibitory signaling pathways play a major role during HIV infection. Although PD-1 expression on HIV-1-specific T cells was first described as a cellular exhaustion marker [9-11], more recent data have emerged indicating that PD-1 expression may instead constitute an attempt to limit immune activation-related immunopathology [12, 13], or simply indicate viral exposure [14, 15]. Furthermore, an increasing body of evidence indicates that PD-1 expression alone may neither be associated with, nor sufficient to characterize an exhausted phenotype, with the co-expression of other inhibitory molecules, such as TIM-3, being essential to define T-cell exhaustion [16, 17].

In chapter 3.2., we investigated the contribution of the PD-1/PD-L1 pathway and TIM-3 expression on T cells to HIV/AIDS immunopathogenesis. Both HIV-1- and HIV-2-infected individuals presented similar patterns of increased PD-1 expression on T cells, with the early and intermediate memory subsets featuring the highest levels of the inhibitory molecule. These data argue against PD-1 expression as an indicator of terminal T-cell differentiation and exhaustion, supporting instead the view that PD-1 up-regulation may indicate T-cell activation upon antigen encounter. In agreement, PD-1^+ T cells were shown to constitute a sizeable proportion of all cycling T cells. This was in marked contrast to TIM-3 expression, which was found to be dissociated from both T-cell cycling and differentiation, identifying a population that was significantly increased in early HIV-1, but not HIV-2, infection. Moreover, the frequency of double-positive PD-1- and TIM-3-expressing T cells was very low in all cohorts, suggesting a differential regulation of PD-1 and TIM-3, and indicating that they are likely expressed

by distinct T-cell subpopulations. Conversely, PD-1 and PD-L1 molecules featured a similar and tightly associated pattern of T-cell expression in all cohorts. Nevertheless, only in the context of HIV-2 infection did the expression of PD-1 and PD-L1 molecules strongly correlate with both the degree of $CD4^+$ T-cell depletion and immune activation. This was despite the undetectable to low levels of viremia found in HIV-2+ patients, and in contrast to what was documented for HIV-1+ individuals, who featured no significant associations between PD-L1 expression and surrogate markers of disease progression.

Overall, these data support a model in which, during HIV-2 infection, a strict and sustained control of PD-L1 expression by T cells takes place, that is somewhat disrupted in the course of HIV-1 disease [18]. In this scenario, a better maintenance of the PD-1/PD-L1 pathway during HIV-2 infection, in the absence of significant TIM-3 expression, could, instead of indicating T-cell exhaustion, help limit immunopathology, thus resulting in slower $CD4^+$ T-cell depletion and, consequently, a more "benign" disease course. These observations have implications for the design of new therapeutic approaches and should be further clarified, namely by the study of PD-1 and PD-L1 expression in lymphoid tissues and the mechanisms underlying their regulation (TCR- and/or γc cytokine-mediated).

The ability to replenish the $CD4^+$ T-cell pool is a main determinant of the course of HIV infection [19]. In chapters 3.3. and 3.4., we aimed to investigate whether the preservation of naïve $CD4^+$ T cells, in particular, could contribute to the characteristically slower rate of HIV-2 disease progression.

As described in chapter 3.3., naïve $CD4^+$ T-cell telomere length was similar in HIV-2-infected individuals and age-matched seronegative controls. This was despite significantly higher levels of cycling naïve $CD4^+$ T cells in HIV-2+ in comparison to seronegative individuals. More importantly, this finding was in marked contrast to what we found in age-matched HIV-1+ individuals, who showed significantly reduced naïve $CD4^+$ T-cell telomere length, relative to age-matched seronegatives, also in the

context of increased cycling. Importantly, our HIV-1 cohort included ART-treated patients who, despite having achieved successful virological suppression, had failed to recover their $CD4^+$ T-cell counts [20]. We found that these so-called HIV-1+ ART-discordants showed the highest frequency of naïve $CD4^+$ T-cell cycling and the most marked reduction in telomere length in comparison to age-matched seronegatives. Interestingly, HIV-1+ ART-discordants have been described to feature impaired thymic function [21, 22]. This is in contrast with previous data from our group supporting a preserved thymic output in the context of HIV-2 infection [23], which possibly contributes to the continuous refill of the $CD4^+$ T-cell pool with cells with long telomeres. Telomere preservation during HIV-2 infection could contribute to a better maintenance of the replicative potential of $CD4^+$ T cells, thereby facilitating $CD4^+$ T-cell compartment homeostasis during chronic disease and promoting slower disease progression.

Together with thymic output, peripheral homeostatic proliferation is considered to play a major role in maintaining the naïve $CD4^+$ T-cell pool [24, 25]. In this respect, we had previously reported that the homeostatic cytokine IL-7 induces a preferential expansion of $CD31^+$ naïve $CD4^+$ T cells, a subset highly enriched in RTE, with no concomitant loss of CD31 expression [26], thereby contributing to maintain the size and diversity of the naïve $CD4^+$ T-cell pool. Furthermore, previous results from our group had also shown that HIV-2+ individuals, who are characterized by a slow rate of $CD4^+$ T-cell depletion and disease progression, presented delayed increases in IL-7 serum levels, coupled with sustained T-cell expression of the IL-7Rα chain, suggesting a maximization of available resources during HIV-2 disease [27]. In order to further explore whether the observed differences in the ability to replenish the $CD4^+$ T-cell pool in HIV-1 and HIV-2 disease could relate to differential responsiveness of naïve $CD4^+$ T cells to IL-7 in the two infections, naïve $CD4^+$ T cells, isolated from individuals in early stage HIV-1 or HIV-2 infection, were stimulated, *in vitro*, with IL-7, as described in sub-chapter 3.4.

Although, at this early stage disease, HIV-1+ individuals featured the highest levels of *ex vivo* naïve $CD4^+$ T-cell proliferation, a significantly higher contribution of

$CD31^+$ cells to the overall pool of cycling naïve $CD4^+$ T cells was observed in the HIV-2 cohort, both in relation to age-matched seronegative and HIV-1+ individuals. Furthermore, naïve $CD4^+$ T cells from HIV-2+ patients were found to have a better sustained proliferative response to IL-7 stimulation, behaving very similarly to those of age-matched seronegative controls. Importantly, this was despite the estimated length of infection being longer in HIV-2+ individuals [5].

Altogether, our data suggest that both preserved telomere length, and enhanced cycling of the RTE-containing $CD31^+$ subset, likely achieved *via* better preserved IL-7 responsiveness, may account for the slow rate of $CD4^+$ T-cell decline during HIV-2 disease. The identification of the mechanisms underlying the maintenance of naïve $CD4^+$ T-cell responsiveness to IL-7 and the differences on IL-7-mediated signaling pathways during HIV-1 and HIV-2 infection, will be of utmost importance, since IL-7 has been proposed to be used as an ART-complementary therapeutic approach [28].

Despite $CD4^+$ T-cell loss constituting the major hallmark of HIV disease progression, several other immune disturbances that occur during chronic infection are important contributors to HIV-related immunodeficiency [29]. Of note, HIV-1 infection has been extensively reported to be associated with a progressive impairment of specific humoral responses and generalized loss of memory B cells, perturbations that are only partially recovered by ART [30]. In chapter 3.5., we sought to address if the memory B-cell compartment was better preserved during HIV-2 infection.

Of note, we found that HIV-2 disease was associated with an overall and irreversible disruption of memory B-cell homeostasis [31], with significant depletion of both unswitched and switched memory B cells being observed, in direct correlation with T-cell activation. This was found even in the absence of circulating viral load, and was not recovered by ART, with treated HIV-2+ patients featuring even more pronounced B-cell imbalances, possibly related to their longer time of infection [5].

It is likely that, despite the low to undectable viremia, significant levels of HIV-2 replication occur in secondary lymphoid organs. Over time, it may result in cumulative, and eventually irreversible, damage to lymphoid tissues, and consequent disruption of germinal centre formation and function, with direct impact on B-cell homeostasis. As data concerning secondary lymphoid organs during HIV-2 infection are still very limited [32, 33], studies regarding the evaluation of lymphoid tissue structure maintenance are key to validating this model.

Of note, it is worth to emphasise that all the aforementioned parameters are inter-related and, thus, their contribution to HIV-2 disease progression in particular, and HIV/AIDS immunopathogenesis in general, cannot be considered individually. A better sustained response to IL-7 stimulation could contribute to an increase in telomere length, due to telomerase induction, and also to PD-1 and PD-L1 up-regulation. On the other hand, it could also possibly facilitate enhanced viral replication in secondary lymphoid tissues, thereby mediating collagen deposition and a progressive slow structural effacement [34, 35]. Further investigation is, therefore, needed, to determine if it is possible to reach an equilibrium point between these inter-connected pathways, such that any benefits are not outweighed by potentially harmful outcomes.

The aim of this project was to address strategies underlying the host's immune response to persistent HIV infection, taking advantage of HIV-2 as a model of naturally-occurring attenuated HIV disease.

Overall, the data presented here support that the slow and more "benign" course that characterizes HIV-2 infection is, in part, related to two main factors.

Firstly, a tight regulation of T-cell activation-inhibition pathways, with over-expression of PD-L1 on both APC and T cells constituting a specific "HIV-2 signature".

Secondly, a better preservation of $CD4^+$ T-cell telomere length and a sustained ability of naïve $CD4^+$ T cells to respond to IL-7 stimulation, which likely impact on the ability to replenish the $CD4^+$ T-cell compartment in HIV-2-infected individuals.

Nevertheless, it is important to note that over the increased time frame of the asymptomatic/chronic phase of HIV-2 infection, exhaustion of immune resources ultimately takes place, as evidenced by the irreversible loss of memory B cells that we documented, even in aviremic HIV-2 disease.

HIV-2 infection is a naturally-occurring attenuated model of HIV disease that remains underrated and not fully explored. This natural model provides an invaluable tool to explore the mechanisms underlying HIV/AIDS immunopathology, with the potential to identify new targets for the development of novel therapeutic approaches.

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