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HIV infection and Follicular Helper T cells: interrogating the SAMHD1 contribution through HIV-2

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Resumo

O vírus da imunodeficiência humana (VIH) continua a representar um problema global de saúde. Atualmente 38,4 milhões de pessoas vivem infetadas com o VIH, tendo sido descritos 1,5 milhões de novos casos em 2021. Apesar de tanto VIH tipo 1 (VIH-1) como tipo 2 (VIH-2) poderem levar ao desenvolvimento da síndrome da imunodeficiência adquirida (SIDA), há uma associação mais frequente do VIH-1 a um trajeto relativamente mais fulminante. O VIH-2, por outro lado, associa-se comumente a um percurso mais insidioso, lento e quase "benigno", sendo que alguns doentes vivem uma vida com reduzidas comorbilidades, carga viral baixa e não chegam a desenvolver SIDA. Tanto VIH-1 como VIH-2 podem sofrer mutações que conferem ao vírus resistência aos tratamentos disponíveis, reforçando a importância dos estudos do VIH não só para minimizar os efeitos adversos dos antirretrovirais, mas também para explorar estratégias para a eliminação das células infetadas.

A infeção pelo VIH tipo 1 (VIH-1) e tipo 2 (VIH-2) apresenta variadas semelhanças, nomeadamente as mesmas vias de transmissão, o alvo de células T CD4 e a utilização de CD4 como recetor principal e de CXCR4 e/ou CCR5 como coreceptores. No entanto, existem algumas diferenças a nível molecular e no estabelecimento e progressão da infeção. A nível molecular, existem diferenças nas suas proteínas, como por exemplo os complexos de glicoproteínas do envelope que facilitam a entrada do vírus na célula são diferentes (gp120 e gp41 no VIH-1, gp105 e gp36 no VIH-2), ainda que equivalentes entre si. As proteínas acessórias entre VIH-1 e VIH-2 também diferem, nomeadamente a ausência da proteína *Vpu* e a presença da proteína *Vpx* no caso do VIH-2 em comparação com VIH-1.

Ainda que seja possível controlar a infeção por VIH com recurso à terapia antirretroviral, esta não erradica as células infetadas pelo vírus, denominadas reservatórios virais, que podem permanecer num estado latente por um período indeterminado, através de mecanismos que ainda não estão totalmente esclarecidos. Apesar de várias células que expressam o recetor CD4 serem reservatórios virais, algumas subpopulações de células T CD4 são considerados mais relevantes. As células T CD4 auxiliares foliculares (Tfh) são um reservatório importante tanto para VIH-1 como VIH-2. Tfh são uma subpopulação

especializada de linfócitos CD4 que se localizam nos órgãos linfoides secundários, nomeadamente na amígdala. Estas células caracterizam-se pela elevada expressão de marcadores celulares como CXCR5, ICOS e PD-1 – marcadores estes que definem a linhagem Tfh –, mas também de Bcl-6, o principal regulador da diferenciação das Tfh. Não só Bcl-6 está envolvido na diferenciação das Tfh, como tem um impacto na suscetibilidade das Tfh à infeção por VIH ao modular fatores de restrição contra estes vírus. Um destes fatores de restrição do hospedeiro é SAMHD1 (do inglês *sterile alpha motif and HD domain-containing protein 1*), que é uma proteína com função de dNTPase, que regula a concentração de desoxiribonucleótidos fosfatados (dNTPs) no citosol, hidrolisando-os, e reduzindo a sua disponibilidade quando a enzima se encontra no seu estado desfosforilado, e, portanto, ativo. Quando fosforilado, SAMHD1 correlaciona-se com níveis mais elevados de dNTPs, traduzindo-se numa diminuição desta atividade enzimática, permitindo a replicação do DNA celular durante a fase S, e assim reduzindo estes níveis em células quiescentes nas quais se registam níveis mais elevados de SAMHD1. Além deste papel no ciclo celular, a redução da disponibilidade de dNTPs inibe a transcrição reversa do VIH e, adicionalmente, estudos mostraram que o SAMHD1 impede a expressão de genes do VIH-1, demonstrando a sua importância enquanto fator de restrição de hospedeiro contra o VIH. O VIH-2 é capaz combater SAMHD1 pela expressão da referida proteína *Vpx* que direciona SAMHD1 para degradação proteossómica, ultrapassando este mecanismo de restrição. Estudos prévios também demonstraram que a *Vpx* confere uma vantagem para o estabelecimento da infeção quando associada ao VIH-1.

Apesar de sabermos que Tfh expressam elevados níveis de Bcl-6 e baixos níveis de SAMHD1, pouco se sabe acerca do papel de SAMHD1 na suscetibilidade das Tfh à infeção pelo VIH. Neste estudo exploramos a capacidade de degradação do SAMHD1 através do VIH-2, conferida pela proteína Vpx, para melhor investigar a interação entre SAMHD1 e HIV em células Tfh. Tendo esses aspetos em conta, colocamos a hipótese de que, através da diminuição dos níveis de SAMHD1 que conferem um entrave à replicação do VIH, o VIH-2 apresentaria algum tipo de vantagem em infetar estes subpopulações celulares de T CD4, nomeadamente no caso de células quiescentes que apresentam os referidos níveis constitutivamente elevados de SAMHD1 e que serão, por esse motivo, menos

suscetíveis à infeção na ausência da *Vpx*. Questionámos, ainda, se esta modulação negativa dos níveis de SAMHD1 pelo VIH-2 impactaria de modo algum o processo de diferenciação das Tfh, comparativamente a células infetadas por VIH-1 e células não infetadas.

Primeiramente, explorámos a infeção por HIV-2 de subpopulações purificadas a partir de células mononucleares isoladas de amígdalas humanas obtidas eticamente, com níveis distintos de SAMHD1, nomeadamente Tfh ($CXCR5^{+}ICOS^{bright}PD\text{-}1^{bright}$) e triple negative (TN, $CXCR5^{neg}ICOS^{neg}PD\text{-}1^{neg}$), posteriormente infetadas com isolados primários de VIH-2 e VIH-1 com tropismos diferentes para os coreceptores, CXCR4 ou CCR5. Analisando os dados gerados, observámos que, apesar de uma maior expressão de SAMHD1, as TN apresentaram elevados níveis de DNA viral total e gag mRNA após 24h de infeção com isolados primários de VIH-2 ou VIH-1, independentemente do coreceptor utilizado. Tanto VIH-1 como VIH-2 conseguem infetar TN e Tfh, indiciando uma ausência de vantagem para o VIH-2 no combate ao SAMHD1. Todavia, os níveis de DNA viral total foram superiores nas Tfh, para ambas as infeções, o que está de acordo com o descrito na literatura. Apesar dos níveis baixos de SAMHD1 em Tfh em comparação com TN, verificámos que existe uma heterogeneidade da expressão de SAMHD1 em células que expressam CXCR5, que não aparenta estar relacionada com a expressão de nenhum marcador característico das Tfh, nomeadamente Bcl-6, OX40, e CD57.

Para avaliar se a infeção pelo VIH-2, e consequentemente a diminuição de SAMHD1 por Vpx, tem impacto na produção de Tfh, realizámos um ensaio com base num protocolo previamente padronizado para a diferenciação *in vitro* de Tfh. Para isso, células T CD4 naïve isoladas a partir de células mononucleares de sangue periférico foram estimuladas e mantidas em cultura com um *cocktail* de citocinas para promover a diferenciação das Tfh. Estas células foram infetadas com isolados primários de VIH-2 e VIH-1 com duplo tropismo para CXCR4 e CCR5, de forma a contornar qualquer viés induzido pelo tropismo para um determinado coreceptor. Realizámos fenotipagem por citometria fluxo e recolhemos *cell pellets* para PCR quantitativo em diferentes dias de cultura. Surpreendentemente, a frequência das Tfh geradas demonstraram-se semelhantes após infeção com VIH-2 e VIH-1, bem como quando comparadas com o controlo não infetado. Mais, as Tfh geradas neste protocolo apresentam expressão semelhante de

diversos marcadores associados às Tfh, indicando um fenótipo similar independentemente da condição de infeção (VIH-2, VIH-1 ou o controlo não infetado).

Concluindo, o VIH-2, apesar de hospedar a *Vpx*, não apresentou vantagem significativa em infetar células T CD4 amigdalinas humanas, independentemente dos seus níveis distintos de SAMHD1. Para além disso, a infeção pelo VIH-2 não modulou a quantidade ou a qualidade das células Tfh geradas com o protocolo diferenciação *in vitro* de Tfh. Estes primeiros dados utilizando isolados primários de VIH-2 incentivam a investigação de mecanismos adicionais no estabelecimento de reservatórios Tfh, bem como as suas implicações na infeção por VIH-2.

Palavras-chave: VIH-2; VIH-1; SAMHD1; Células T Auxiliares Foliculares;

Abstract

Antiretroviral therapy does not eradicate HIV-infected cells, also known as viral reservoirs. Follicular helper CD4 T cells (Tfh), a specialized subset of CD4 lymphocytes, have been shown to be important HIV reservoirs, for both HIV type 1 (HIV-1) and type 2 (HIV-2). Bcl-6, the regulator of Tfh differentiation, has been shown to impact cellular susceptibility of Tfh to HIV infection by modulating the expression of HIV restriction factors. One of these restriction factors is SAMHD1 which has been shown to impair HIV-1 gene expression. HIV-2 can counteract SAMHD1 due to the expression of Vpx. Here we explore that ability to further investigate the interplay of SAMHD1 and HIV in Tfh. Firstly, we explored HIV-2 infection of sort-purified subsets from human tonsils with distinct levels of SAMHD1: Tfh ($CXCR5^{+}ICOS^{bright}PD\text{-}1^{bright}$) and triple negative (TN, $CXCR5^{neg}ICOS^{neg}PD\text{-}1^{neg}$). Despite higher SAMHD1 expression, TN also presented high levels of total HIV DNA and gag mRNA upon 24h infection with either HIV-2 or HIV-1 primary isolates. Nevertheless, total HIV DNA levels were higher in Tfh in both infections. Then, we asked whether HIV-2 infection biased Tfh generation using a standardized protocol for *in vitro* Tfh differentiation from naïve cells. Surprisingly, the frequency of the generated Tfh and their detailed phenotype were remarkably similar upon effective infection with HIV-2 or HIV-1 primary isolates. In conclusion, HIV-2, despite harbouring Vpx, did not feature a significant advantage to infect tonsillar CD4 T cells, irrespectively of their SAMHD1 levels, and did not modulate *in vitro* Tfh differentiation. These first data using HIV-2 primary isolates prompt the investigation of additional paths in the establishment of Tfh reservoirs, and their implications for the benign course of HIV-2.

Keywords: HIV-2; HIV-1; SAMHD1; Follicular Helper T cells.

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Preface

In order to highlight the importance of HIV studies and have a professionally experienced overview of the status of HIV infection, disease progression and treatment in Portugal, I conversed with Doctor Teresa Branco, member of the direction of the Portuguese Association for the Clinical Study of AIDS, and Doctor Robert Badura, Senior Consultant for Infectious Diseases at the Department for Infectious Diseases in the University Hospital de Santa Maria, both acclaimed health professionals with many years of experience in dealing with HIV.

When inquired about the general scene of HIV infection in Portugal, Doctor Teresa pointed out to a transition from a very likely death-sentence to a now rather chronic illness, paired to the development of novel and more effective pharmacological approaches that have less side effects. However, the disease's chronicity also presents challenges, namely the establishment of comorbidities impacting patients' quality of life that must not remain unaddressed. Doctor Robert, on the other hand, reported a view in which new infection cases are still regularly documented and that patients are still dying, affirming that the situation has not yet stagnated. He also stated that there are still numerous late diagnostics in the cases of heterosexual patients, which may not be accelerating but are nonetheless increasing. Concerning the main barriers to the treatment of HIV in Portugal, Doctor Teresa promptly identified the stigma that surrounds HIV as a focal point for the lack of sufficient screening. Individuals tend to not test themselves for the fear of experiencing exclusion, coupled with many other social, cultural and logistics issues that further aggravate the scenario, such as reduced access to pre-exposure prophylaxis (PrEP) which is only available in reference centers, imposing day-to-day difficulties for the average person, and particularly in specific risk groups such as sex-workers. Besides the stigma, Doctor Robert designated as major factor the lack of disseminated knowledge and literacy in the general community when it comes to HIV. Another crucial factor is the lack of accessibility associated with the limitations of the established risk groups leading to the exclusion of patients that keep being underdiagnosed. Doctor Robert mentioned that screening should even be widely available at pharmacies, easing the access to the test. Describing the clinical

presentations of HIV-2 infection, Doctor Robert notes that the disease should be considered slower than HIV-1 rather than having a benign course, mentioning that some patients carry the disease for periods as long as two decades without presentation of almost any signs or symptoms while others aggravate and never achieve an ideal immunologic state. Doctor Teresa also describes clinical variability within HIV-2-infected individuals, identifying that CD4 T cell counts can drop drastically when viral load becomes detectable with the deterioration of health condition of some patients while other patients present low CD4 counts even though having undetectable viral load. She also stated that HIV-2 diagnostic is strongly associated with opportunistic screening in asymptomatic patients, providing the example of pregnancy, where testing sometimes leads to the diagnostic of both partners, but also through the identification of opportunistic diseases such as oropharyngeal candidiasis and tuberculosis, associated with progressed infection.

When it comes to the therapeutic difficulties for HIV-2, both regarded how differently HIV-2 mutates in comparison to HIV-1 and develops resistance to the available treatment options more tailed for HIV-1, possibly culminating in an untreatable disease. Concerning the major challenges handled by these professionals, Doctor Teresa listed the incapacity to provide efficient global healthcare and social support, which she attributes to factors as the decrease in consultation times, a decline in the specialization of nurses and pharmaceutical professionals, conditioning a failure in comorbidity prevention. Doctor Robert additionally remarks the lack of sufficient screening and alerts that health professionals should be especially sensibilized to request the test whenever necessary. Moreover, he appoints that access to society should be polyvalent, multicultural, and open towards all realities.

Overall, these comments further support the value and importance of HIV studies, namely in the case of HIV-2 given its particularities, paving the way for future discoveries in hope to overcome any and every obstacle imposed by these pathogens.

Abbreviations

AGS – Aicardi-Goutières syndrome

AIDS – Acquired immunodeficiency syndrome

ART – Antiretroviral therapy

Bcl-6 – B-cell lymphoma 6

BSL3 – Biosafety Level 3

R5 - CCR5 – C-C chemokine receptor type 5

CCR7 – C-C chemokine receptor type 7

CD4 – Cluster of differentiation 4

cGAS – Cyclic GMP-AMP synthase

X4 - CXCR4 – C-X-C chemokine receptor type 4

CXCR5 – C-X-C chemokine receptor type 5

DC – Dendritic cell

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

dNTP – Deoxynucleoside triphosphate

env – HIV envelope glycoprotein

FBS – Fetal bovine serum

FCS – Flow cytometry standard

Gag – Group-specific-antigen protein

GC – Germinal center

gp – Glycoprotein

HIV – Human immunodeficiency virus

HIV-1 – Human immunodeficiency virus type 1

HIV-2 – Human immunodeficiency virus type 2

ICOS – Inducible T cell costimulatory molecule

ICOSL – ICOS Ligand

IFN – Type 1 Interferon

IL – Interleukin

LTR – Long terminal repeat

MHC-II – Major histocompatibility complex class II

mRNA – Messenger RNA

Nef – Negative factor

NF-kB – Nuclear factor kappa-light-chain-enhancer of activated B cells

PBMC – Peripheral blood mononuclear cells

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

PD-1 – Programmed cell death protein 1

PD-L1 - Programmed cell death protein ligand 1

Pol – DNA polymerase gene

PrEP – Pre-exposure prophylaxis

RNA – Ribonucleic acid

RT – Reverse Transcriptase

SAMHD1 – Sterile alpha motif and HD domain-containing protein 1

SIDA – Síndrome da imunodeficência Humana

SIV – Simian immunodeficiency virus

STAT3 – Signal transduces and activator of transcription 3

STING – Stimulators of interferon genes

Tat – Trans-activator of transcription

TCR – Long terminal repeat

Tfh – Follicular helper T cell

TGF-β – Transforming growth factor-beta

TMNC – Tonsillar mononuclear cells

TN – Tripple negative

UMAP – Uniform Manifold Approximation and Projection

Vif – Viral infectivity factor

VIH – Vírus da imunodeficiencia Humana

VIH-1 – Vírus da imunodeficiencia Humana tipo 1

VIH-2 – Vírus da imunodeficiencia Humana tipo 2

Vpr – Viral protein R

Vpu – Virion protein U

Vpx – Viral protein X

Introduction

1. Human Immunodeficiency Virus

1.1. Epidemiology

According to UNAIDS's 2022 Global AIDS update, 1.5 million adults and 160,000 children had been newly infected with Human Immunodeficiency Virus (HIV) in 2021, and an estimated 38.4 million adults were living with HIV. In that same year, 552,000 adults and 98,000 children have died of Acquired Immunodeficiency Syndrome (AIDS) (*Global HIV & AIDS Statistics — Fact Sheet*, n.d.).

In Portugal, since 1938 and until 2021, 64257 cases of HIV infection were reported of which 23399 developed AIDS, a total of 15555 deaths are documented. A number of 1803 new cases of HIV infection were notified in 2020 and 2021, 71.8% of those cases corresponded to male teens and adults. Heterosexual transmission continues to be more frequent with 51.8% was observed, but 56.0% of the new cases among men were linked to men who have sex with men (*Relatório Infeção Por VIH Em Portugal – 2022 - INSA*, n.d.).

1.2. HIV pathogenesis

The Human Immunodeficiency Virus (HIV) is a retrovirus that can be grouped in two types, HIV-1 and HIV-2. HIV-2 is characterized by a longer asymptomatic phase, a slower decrease in CD4 T cells, and lower viral levels in the blood, when compared to HIV-1. These advantages are associated with an overall lower mortality; however HIV-2 infected patients can still develop AIDS and succumb to the disease (AK Kapoor & Padival, n.d.; Esbjörnsson et al., 2019; Soares et al., 2011). Studies have shown that although HIV-2 infection can correlate with higher CD4 T cell count, once the decrease reaches numbers bellow 200 cells/mm^3, the mortality rates are comparable to those of HIV-1 with similar cell counts (Campbell-Yesufu & Gandhi, 2011). Although HIV-2 can be treated with antiretroviral therapy (ART) mainly developed to combat HIV-1, several cases of drug resistance through viral mutations have been reported as they have been for HIV-1 (Requena et al., 2017). These resistances can lead to viral rebound (Requena

et al., 2017) which translates to a current need of finding new optimized strategies for the control of both HIV-2 and HIV-1 infections.

Due to its close ties with Portuguese-speaking African countries, namely Guinea-Bissau and Cape Verde, Portugal present a notably high prevalence of HIV-2 infection when compared to its European counterparts (Visseaux et al., 2016).

1.3. HIV genome and cell cycle

These viruses present a genome comprised of two strands of identical copies of RNA and can be identified by the presence of specific structural genes: *gag, pol* and *env* (Figure 1) (Cohen et al., 2008; Fanales-Belasio et al., 2010; Ferguson et al., 2002). Despite their molecular similarities, several key differences can be found between HIV type 1 and type 2. While HIV-1 envelope glycoprotein complex is comprised of gp120 along with gp41, HIV-2 on the other hand has gp105 and gp36 glycoproteins (Fanales-Belasio et al., 2010; Goodsell, 2015; Valadés-Alcaraz et al., 2022). HIV-1 encodes accessory proteins, namely viral infectivity factor (Vif), viral protein R (Vpr), negative regulatory factor (Nef) and virion protein U (Vpu), playing different roles from counteraction of host restriction factors, modulation of immune responses to enhancement of viral infectivity. Although the other proteins are expressed by HIV-2, Vpu is not present in this virus. Of note, HIV-2 harbours another accessory protein called viral protein X (Vpx) which is known to counteract a specific host restriction factor, SAMHD1, which will be further addressed below(Mohamed et al., 2021).

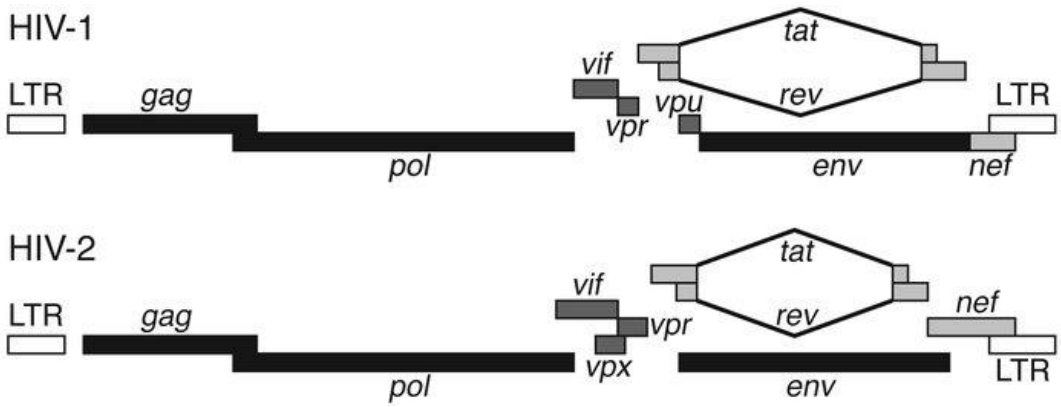

Figure 1. Structure of HIV-1 and HIV-2 genomes. From *Cohen et al., 2008*

Two phases can be defined in HIV replication cycle: the early phase and the late phase. The first addresses the recognition of the target cell and stretches to the integration of the viral DNA into the host cell chromosome, the latter refer to all processes after integration, as seen on Figure 2 (Fanales-Belasio et al., 2010; Goodsell, 2015; *HIV Replication Cycle | NIH*, 2018; Turner & Summers, 1999).

The infection starts with viral binding via glycoprotein gp120 to receptor CD4, leading to conformational changes that promote attachment to chemokine coreceptors, mainly CCR5 or/and CXCR4. Fusion is triggered by these additional conformational changes, allowing the entry of viral core to the cytoplasm to undergo uncoating. This step involves core disassembly by dissociation of the capsid which is critical for the progress of reverse transcription. Single-stranded viral RNA is subsequently transcribed by viral RT into double-stranded DNA, which will be delivered into the nucleus to be incorporated into the host cell genome. At this step, integrated viral DNA (or provirus) can become latent or become transcriptionally active. If transcriptionally active, viral DNA is transcribed and viral mRNA transcripts are exported to the cytoplasm to be translated into proteins and to be incorporated into new particles and genomic RNA. The viral proteins and RNA are then assembled into viral particles followed by budding from the plasma membrane of the cell and maturation to become infectious (Ferguson et al., 2002; Turner & Summers, 1999).

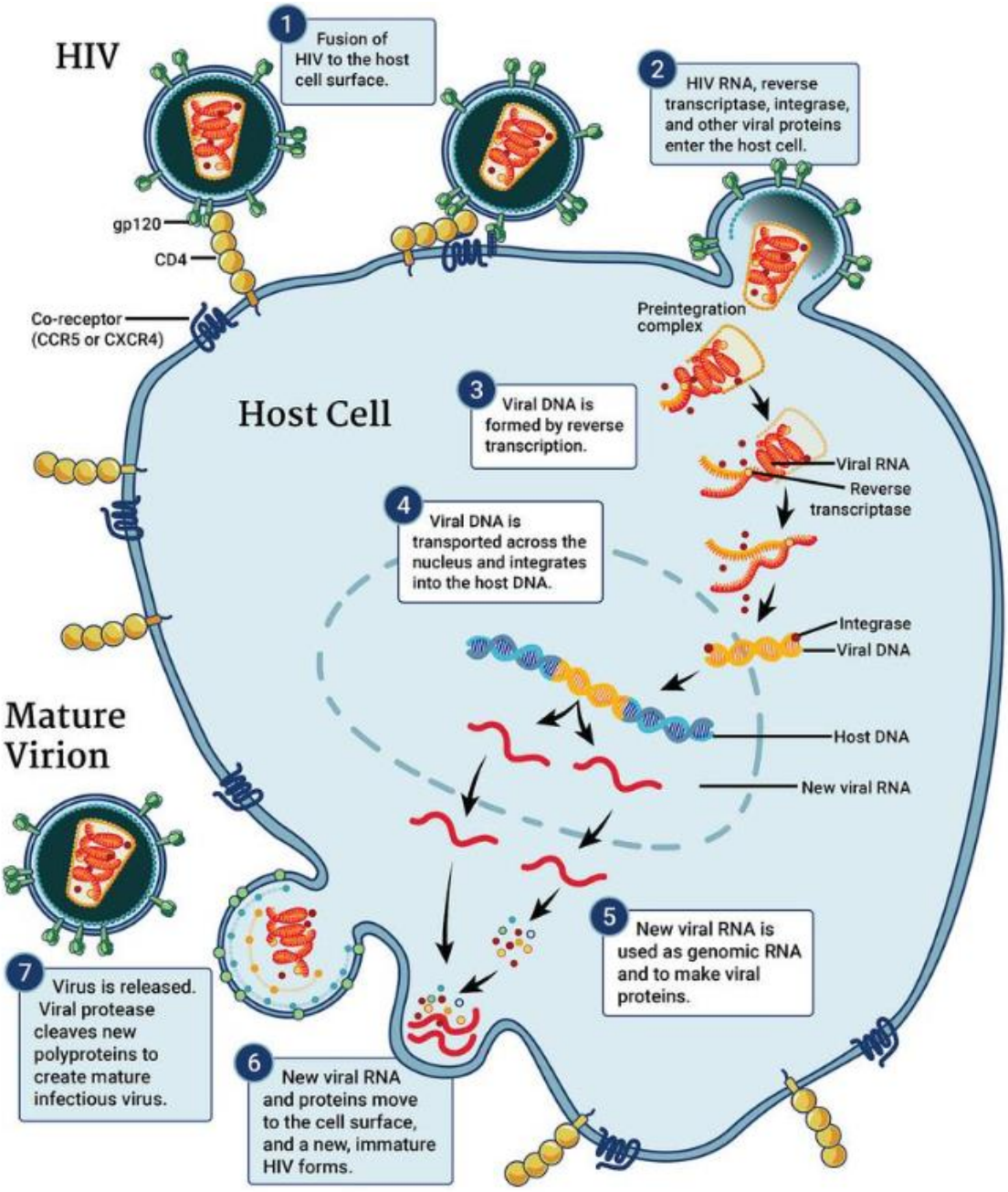

Figure 2. Overview of HIV life cycle. From *NIH*

1.4. Establishment of Viral reservoirs

As mentioned above, HIV possesses the ability to remain latent upon infection. Latently-infected cells or viral reservoirs constitute one of the main barriers for HIV cure, as ART does not eradicate these HIV infected cells. Moreover, these reservoirs can remain latent for extended periods of time and might be responsible for viral rebound upon ART interruption. Although several cells bearing CD4 receptor have been demonstrated to be HIV reservoirs, such as macrophages or dendritic cells, the major subset is considered to be resting CD4 T cells (Aid et al., 2018; Chen et al., 2022; Pedro et al., 2019). Although the main mechanism for the establishment of latent CD4 T cells is not yet particularly understood, it is known that one the mechanisms is direct infection of resting T cells even though comparatively activated cells are preferably and more easily infected (Pedro et al., 2019). Follicular helper T cells have been shown to be key reservoirs for both HIV-1 and HIV-2, capable of supporting replicative infection(Godinho-Santos et al., 2020; Perreau et al., 2013).

2. Follicular Helper T cells

Follicular helper CD4 T cells (Tfh) are a specialized subset of CD4 lymphocytes that can be found in the B cell follicles of secondary lymphoid organs (SLOs), such as the tonsils and lymph nodes. Tfh promote antibody-mediated immune responses, by contributing to the survival, activation, and maturation of germinal center (GC) B cells, selecting high-affinity B cell receptors during the GC reaction (Breitfeld et al., 2000; Crotty, 2014). Tfh can be characterized by the high expression of lineage-defining markers such as the C-X-C chemokine receptor type 5 (CXCR5), programmed cell death protein 1 (PD-1), inducible T cell costimulatory molecule (ICOS), but also by the expression of Tfh master transcription regulator, Bcl-6 (B-cell lymphoma 6)(Qi et al., 2014). Bcl-6 enhances CXCR5 expression, which dictates the commitment of primed CD4 T cells towards B cell follicles, while PD-1/PD-L1 and ICOS/ICOSL interaction further contributes to the upregulation of CXCR5 and Tfh commitment (Jogdand et al., 2016; Kitano et al., 2011; Shi et al., 2018; Yu et al., 2009).

2.1. Bcl-6 as major regulator in:

2.1.1 Tfh differentiation process

Upon antigen presenting by dendritic cells (DC), naïve CD4 T cells are primed via T cell receptor (TCR) and major histocompatibility complex class II (MHC-II) with additional stimulation through co-stimulatory molecules, such as ICOS, and cytokines such as interleukin 6 (IL-6) and IL-12 (Figure 3)(Crotty, 2014; Jogdand et al., 2016). All these signals trigger Tfh cell differentiation program through the up-regulation of Bcl-6 (Olatunde et al., 2021; Yu et al., 2009). TGF-β together with IL-12 and IL-23 are also involved in Tfh cell program initiation through STAT3, in addition to IL-1β and IL-6 (Schmitt et al., 2014; Wu et al., 2018). These activated CD4 T cells begin to downregulate CCR7 and upregulate CXCR5 and Bcl-6, which by acting along other transcription factors, lead to the commitment of the cells to Tfh-lineage. These cells then migrate to the T-B border, where receptor-ligand interactions such as ICOS/ICOSL, PD-1/PD-1L and CD40L/CD40 occur between Tfh and B cells (Crotty, 2014; X. Ma et al., 2021; Wu et al., 2018). Tfh-B cell interaction allows the formation and maintenance of GC in B cell follicles, leading to B to mature into short-lived extrafollicular plasmablasts or relocate into follicles (Fan et al., 2015; Liu et al., 2013). As Tfh progress to the GC, they upregulate

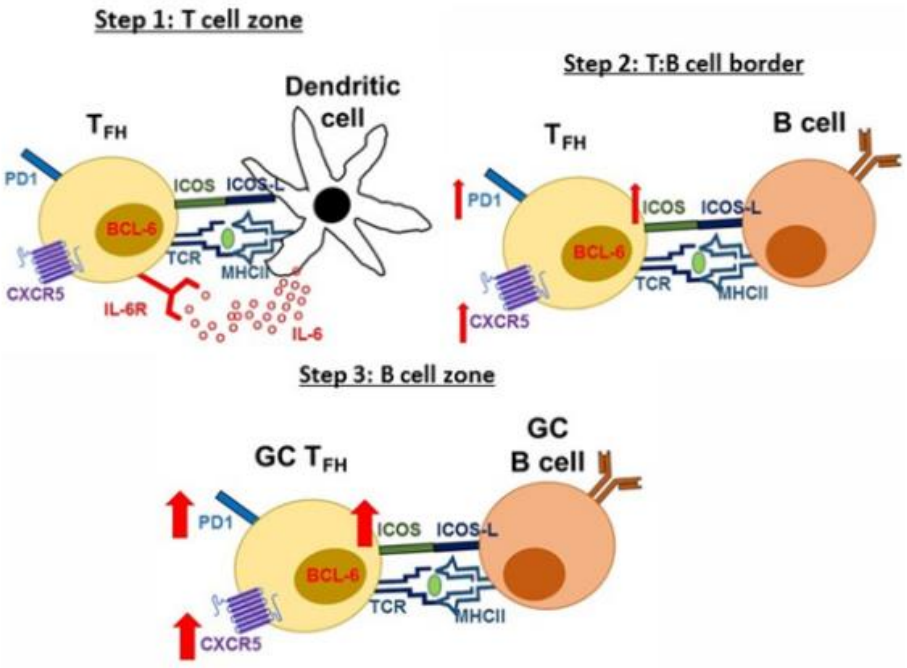

Figure 3. Tfh differentiation. From the *British Society of Immunology*

other defining markers such as CD40 Ligand (CD40L), OX40, CD57 and CD69, along with Bcl-6 (Deenick & Ma, 2011; C. S. Ma et al., 2012; Padhan et al., 2021)

2.1.2. Tfh susceptibility to HIV

The aforementioned master transcription regulator, Bcl-6, also plays a role in cellular susceptibility to infection. In fact, studies have demonstrated that Bcl-6 inhibition resulted in lower HIV-replication, as well as T cell proliferation (Cai et al., 2019). Moreover, Bcl-6 has been shown to mediated cellular susceptibility to HIV infection, also because it can modulate host restriction factors, such as SAMHD1 (Amet et al., 2017; Cai et al., 2019).

3. SAMHD1

The sterile alpha motif and HD domain-containing protein 1, or SAMHD1, is a host restriction factor that inhibits HIV reverse transcription by reducing the intracellular deoxynucleoside triphosphate (dNTP) pool as illustrated in Figure 4 (Hrecka et al., 2011; Laguette et al., 2011; Lahouassa et al., 2012). Nevertheless, SAMHD1 can be counteracted by HIV-2 protein Vpx by mediating its proteasomal degradation (Hrecka et al., 2011). Several studies have demonstrated that Vpx can enhance HIV-1 infection in macrophages, associated with an increase of intracellular dNTPs(Baldauf et al., 2017a; Descours 2017).
More recently, SAMHD1 has been found to also suppresses HIV-1 LTR-driven gene expression(Antonucci et al., 2018).

Due to its dNTP hydrolase activity, SAMHD1 has key functions in cell cycle. Indeed, its C-terminal T592 residue phosphorylation is correlated with higher dNTP levels preceding the S-phase DNA replication, while SAMHD1 dephosphorylated form is associated with a reduced dNTP pool, which predominates in cells in non-cycling G0/quiescent state (Mauney & Hollis, 2018; Schott et al., 2018). It also reduces cell metabolism and proliferation in THP-1 cells (Bonifati et al., 2016). SAMHD1 is also linked to RNA homeostasis, where it prevents an accumulation of cellular RNA that ultimately triggers antiviral type I interferon (IFN) response. An example is Aicardi-Goutières syndrome

(AGS), a disorder characterized by a constant activation of IFN (Coggins et al., 2020; Maharana et al., 2022).

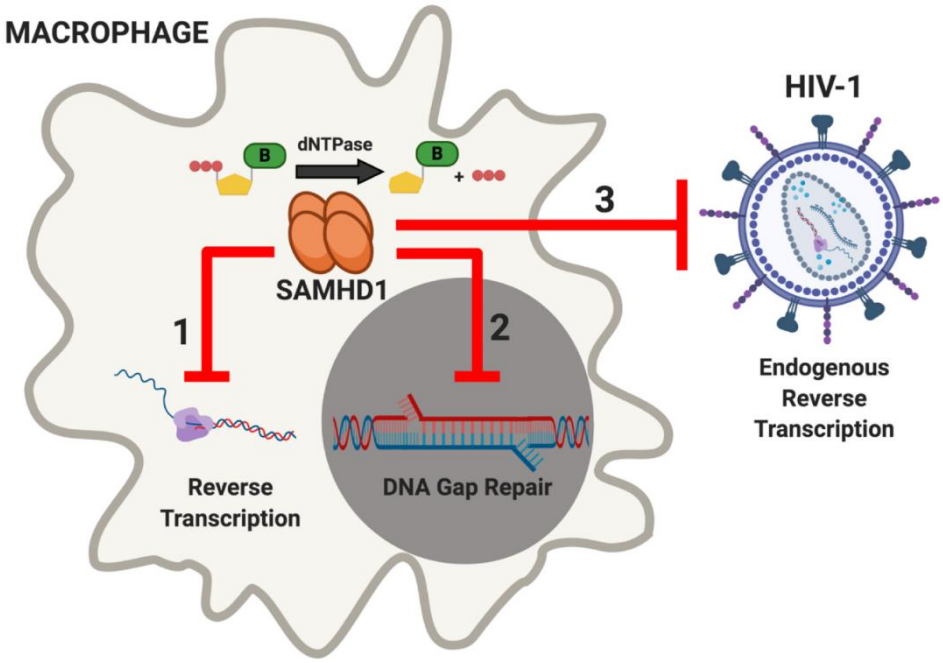

Figure 4. SAMHD1 function in macrophages. SAMHD1 induces dNTP pool reduction and blocks RT in the cytosol (1), gap repair in the nucleus (2) and endogenous RT that occurs extracellularly (3). From *Coggins et al., 2020*

As described above, SAMHD1 can be downregulated through Bcl-6, the master regulator of Tfh. Of note, one study has shown that Bcl-6 can lead to a decrease in phosphorylated SAMHD1, a consequent reduction in T cell activation and proliferation, and a hindering in HIV replication both in peripheral blood and Tfh (Cai et al., 2019).

Aims and Hypothesis

Tfh are important HIV-1 and HIV-2 reservoirs. It is known that that the specific subset of CD4 T cells express high levels of Bcl-6 and consequently lower levels of SAMHD1, while quiescent CD4 T cells, namely TN, express high levels of this protein that restricts HIV infection. Therefore, it is logical to assess if these expression patterns contribute to the documented high susceptibility of Tfh to HIV infection. Since studies have shown that Vpx can enhance HIV-1 infection by mediating the degradation of SAMHD1, we hypothesized that HIV-2, by expressing Vpx, should present with any kind of advantage for the viral infection in Tfh.

Thus, the main objective of this study was to investigate the interaction between SAMHD1 and HIV-2 in Tfh. To accomplish this goal, we firstly intended to investigate the potential influence of SAMHD1 levels in cellular susceptibility to HIV and the establishment of viral reservoirs by the infection of two sort-purified CD4 T cell subsets from human tonsils with distinct levels of SAMHD1. Secondly, we aimed to assess the impact of SAMHD1 in Tfh differentiation using HIV-2 infection, and subsequently SAMHD1 downmodulation through Vpx, in a previously optimized *in vitro* Tfh differentiation protocol.

Methods

Human samples

Tonsils were obtained through routine paediatric tonsillectomy in collaboration with the ETN department of Centro Hospitalar Universitário Lisboa Norte (CHULN) Lisbon, Portugal) and buffy-coats from Instituto Português do Sangue e Transplantação (IPST). The study was conducted under the approval of the Ethical Board of the CHULN/Faculdade de Medicina da Universidade de Lisboa/ Centro Académico de Medicina de Lisboa, and samples collected after written informed consent from participants or their legal representatives.

Cell isolation

Tonsillar mononuclear cells (TMNCs) were recovered through tissue dispersion by maceration of human tonsil specimens which were firstly placed in RPMI 2%FBS [RPMI-1640 medium (RPMI, Gibco) supplemented with 2% fetal bovine serum (FBS, Gibco)] in a petri dish for tonsil preparation. After removing cauterized parts and dissecting the tissue for maceration through a 70um nylon membrane cell strainer, the cells were collected in a 50mL falcon tube containing RPMI 2%FBS. Using Ficoll-Paque Plus (GE Healthcare) density gradient, mononuclear cells were isolated after centrifugation at 2100rpm for 15min. The mononuclear cells were then washed twice with RPMI 2%FBS, centrifuging at 1600rpm for 10 minutes, posteriorly counted in a Neubauer chamber, using Trypan Blue to exclude dead cells. Part of the TMNCs were used for phenotyping and the remnant cells were resuspended in a freezing medium composed of FBS supplemented with final 7.5% Dimethyl sulfoxide (DMSO, Sigma) and stored in liquid nitrogen.

CD4 T cells were isolated from thawed TMNCs which were first washed with RPMI 20% FBS, counted with trypan blue and resuspended in RPMI medium containing 10% fetal bovine serum, 1% L-glutamine 1% Pen-Strep, 1x sodium pyruvate, 1x non-essential amino acids and 0,1% Gentamicin (all reagents from Gibco) (RPMI complete medium) in order to be cultured overnight at a density of 10 million cells/ml. The TMNCs were then magnetically enriched using the untouched Human CD4 T-Cell Isolation Kit, (MojoSort; BioLegend). Subsequently, isolated CD4 T cells were counted in acetic acid, separated

into tubes for cellular staining and sorted using a FACS ARIA (BD Biosciences) as illustrated in Figure 5 to obtain Tfh ($CXCR5^{+}ICOS^{bright}PD\text{-}1^{bright}$) and quiescent triple negative (TN - $CXCR5^{neg}ICOS^{neg}PD\text{-}1^{neg}$) cells.

Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats and diluted in a proportion of 1:2 of Phosphate-buffered saline (PBS) before performing Ficoll density gradient separation. After centrifugation at 2100rpm for 20 minutes, the cells were collected using a Pasteur pipette to a new falcon with PBS, washed three times centrifuging at 1600rpm and counted in a Neubauer chamber. Naïve CD4+ T cells were purified from PBMCs by negative selection using MojoSort Human CD4 T Cell Isolation Kit (BioLegend), according to manufacturer instructions (purity > 95%, n=3).

Flow cytometry

TMNCs were thawed and washed to remove DMSO, counted using trypan blue and resuspended in RPMI complete medium to be incubated overnight at 37°C 5%CO_2 at density of 10 million cells/mL. In the following day, cells were collected, counted, and stained for surface and intracellular antigens as previously described using antibodies listed in Table 1 (Godinho-Santos et al., 2020; Nunes-Cabaço et al., 2014), as well as LIVE/DEAD Fixable Dead Cell Stain (Invitrogen). When needed, intracellular staining was performed after surface staining using eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set kit, according to manufacturer's instructions. Samples were acquired in a Cytek Aurora (Cytek Biosciences) 3L configuration (16V-14B-8R) and analysed using FlowJo v10 software.

Unsupervised analysis was further performed in selected populations in R version 4.2.2 with Bioconductor version 3. The FCS files were imported into R and the data was transformed using arcsinh (Hahne et al., 2009); flowCore (Hahne et al., 2009); flowVS (Azad et al., 2016); and then all markers except SAMHD1 and CXCR4 were normalized based on warping functions computed on high-density region landmarks utilizing the flowStats package (Hahne et al., 2023). With the aim of visualizing the high dimensional data, a UMAP was applied to the dataset using the CATALYST package (Crowell et al., 2023) performing Uniform Manifold Approximation and Projection for Dimension Reduction training the model to use the same number of events from each sample (5000 events/sample).

Table 1. List of Antibodies used in Flow Cytometry

Marker	Clone	Fluorochrome	Source
Bcl-6	K112-91	AF647	BD
CCR5	2D7/CCR5	PE-Cy7	BD
CCR7	150503	FITC	R & D Systems
CD127	A019D5	AF700	BioLegend
CD16	eBioCB16	PE	eBioscience
CD19	HIB19	PE-Cy7	eBioscience
CD25	A2A3	APC	BD
CD25	BC96	PE-Cy5	BioLegend
CD3	UCHT1	BV480	BD
CD3	OKT3	eFluor450	Invitrogen
CD4	RPA-T4	BV711	BioLegend
CD4	RPA-T4	PE-Cy5.5	Invitrogen
CD45RA	HI100	AF700	BioLegend
CD45RA	HI100	BV650	BioLegend
CD45RO	UCHL1	BV570	BioLegend
CD45RO	UCHL1	APC	BD
CD56	C5.9	PE	Cytognos
CD57	TB01	eFluor450	Invitrogen
CD69	FN50	BV785	BioLegend
CD8	RPA-T8	PE-Cy5.5	eBioscience
CD8	RPA-T8	BV711	BioLegend
CXCR4	12G5	PE-Cy5	BioLegend
CXCR5	J252D4	PE-Dazzle694	BioLegend
Foxp3	PCH101	PE	Invitrogen
HLA-DR	L243	BV650	BioLegend
HLA-DR	G46-6	V500	BD
ICOS	C398.4A	BV510	BioLegend
OX40	ACT35	BV421	BioLegend
PD-1	EH12.2H7	BV605	BioLegend
SAMHD1	l19-18	FITC[a]	Merck
TCRγδ	11F2	PE-Cy7	BD

[a] Purified antibody conjugated in house with FITC.

Viral stocks

Viruses used in this thesis were previously produced in Biosafety Level 3 (BSL3) laboratory using isolated PBMCs and infections particles/ml (PFU/ml) were assessed by infection of reporter cell line TZM-Bl that holds an HIV Tat-responsive LTR promoter driving the expression of β-galactosidase and luciferase.

HIV-2 and HIV-1 infection of tonsillar purified subsets

HIV-1 and HIV-2 primary isolates were selected based on CCR5- (R5-) or their CXCR4- (X4-) usage, namely: HIV-2_{R5} 60415K; HIV-2_{X4} 20.04; HIV-1_{R5} 92US660; HIV-1_{X4} 92HT599 (from NIH AIDS Reagent Program, except 20.04 which was provided by Nuno Taveira as described (Godinho-Santos et al., 2020; Nunes-Cabaço et al., 2014)). Briefly, 2.5×10^5 Tfh or TN were infected with primary isolates (940 PFU/10^6 cells) for 3h with 3 µg/mL Polybrene (Sigma), washed, and cultured for 24h in 96-well plate (COSTAR) with a density of 2.5×10^6 cells/mL in complete medium at 37°C/5% CO2. Cells were collected at 24h for cell pellets storage at -80^{o}C for quantification of viral readouts.

In vitro Tfh differentiation assay in the absence and presence of HIV-2 or HIV-1

Purified circulating naïve CD4 T cells were labelled with Cell Trace Violet^{TM} (CTV, Invitrogen), and TCR stimulated with DynaBeads Human T-activator CD3/CD28 (Life Technologies) overnight in a beads:cell proportion of 1:4. After washing to remove the stimuli, cells were incubated for 3h with dual-tropic viruses HIV-1 92/US/723 or HIV-2 CBL-23 primary isolates (from NIH AIDS Reagent Program) (940 PFU/10^6 cells) with 3 µg/mL Polybrene (Sigma) in BSL3. Cells were subsequently washed and cultured with immobilized anti-CD3 (5µg/ml Clone OKT3, eBioscience) and soluble anti-CD28 (1µg/ml Clone CD28.2, eBioscience) and supplemented with an optimized mix of human recombinant cytokines known to promote Tfh differentiation (Schmitt et al., 2014), namely IL-12 [10ng/ml] + IL-1β [10ng/ml] + IL-6 [25ng/ml] + TGF-β [5ng/ml] (all from Peprotech). In parallel, stimulated cells and unstimulated cells were cultured with IL-2 [10U/ml] and used as controls. After 72h of culture (day 5), TCR stimuli was removed, and cells were resuspended in fresh medium supplemented with either the cytokine mix or IL-2 and maintained in culture for additional 48h (day 7). Cells were collected at days

5 and 7 for immunophenotyping by flow cytometry and for cell pellets storage at -80°C for quantification of viral readouts.

Quantification of total viral DNA, gag mRNA and SAMHD1/Bcl-6 transcriptional levels

Cell pellets collected after 24h of infection of tonsillar CD4 T subsets and throughout the *in vitro* Tfh differentiation assay were used for DNA and RNA extraction using the AllPrep RNA/DNA Micro kit (QIAGEN), according to manufacturer's instructions.

For total HIV-1 and HIV-2 DNA quantification, quantitative real-time PCR (qPCR) was performed using sets of primers already validated in the lab (see table 2). Both gag and CD3 were amplified in the same sample in order to assess number of gag copies per million of cells. Reactions contained 1X TaqMan® Gene Expression Master Mix (Applied Biosystems), 400 nM each primer, 250 nM probe and 100ng of template DNA, in a 20 µl volume. After initial incubations at 50°C for 2 min and 95°C for 2 min, 50 cycles of amplification (95°C x 30s; 60°C x 30s; 72°C x 2min;) were performed using an ABI 7500 Real-Time PCR System (Applied Biosystems).

For gag mRNA quantification, 80 ng of total RNA was used to synthesize complementary DNA using oligo(dT)20 and NZY First-Strand cDNA Synthesis Kit, separate oligos (NZYtech). For HIV-1 and HIV-2 mRNA quantification, primers and probes used are listed in Table 2. For quantification of *β-actin, samhd1 and bcl-6* mRNAs, a step of pre-amplification was performed with TaqMan PreAmp master mix (Applied Biosystems) according to manufacturer's instructions, using commercial TaqMan primers and probes mixes (Thermo Fischer Scientific, SAMHD1 Assay ID: Hs00210019_m1; Bcl-6 Assay ID: Hs00153368_m1, GAPDH Assay ID: Hs02786624_g1). Reactions contained 1X TaqMan® Gene Expression Master Mix (Applied Biosystems), 1x each gene assay and dilution 1:5 to 1:20 of pre-amplified cDNA products, in a 6 µl volume. After initial incubations at 50°C for 2 min and 95°C for 10 min, 40 cycles of amplification (95°C x 15s; 60°C x 60s) were performed using an ABI 7500 Real-Time PCR System (Applied Biosystems).

Table 2. Primers and Probes used to quantify cell-associated viral burden.

Primer/Probe	Gene	Sequence
Forward	CD3	5'-AGGGCAAAATGGAGGCTCTTA-3'
Reverse	CD3	5'-TCTCCTCCATGGGACACTGTT-3'
Probe	CD3	5'- VIC-CTCTCTAGCAGAGAACAGT-MGB-3'
Forward	HIV-1 gag	CGAGAGCGTCAGTATTAAGC
Reverse	HIV-1 gag	AGCTCCCTGCTTGCCCATAC
Probe	HIV-1 gag	5'-FAM-CCCTGGCCTTAACCGAATT-MGB
Forward	HIV-2 gag	CGCGAGAAACTCCGTCTTG
Reverse	HIV-2 gag	GCTGCCCACACAATATGTTTTA
Probe	HIV-2 gag	5'- FAM-CCGGGCCGTAACCT-MGB

Statistical analysis

Wilcoxon signed-ranked test was used to compare paired datasets. Data were analyzed with R version 4.2.2., using the *ggplot2*, *dplyr* and *rstatix* packages for data visualization and analysis. GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA) was also used, recurring to Benjamini Krieger and Yekutieli test. A p value <0.05 was considered statistically significant.

Results

HIV-2 and HIV-1 infection of purified CD4 T cells from human tonsils

To assess whether SAMHD1 levels had an impact in cellular susceptibility to HIV and viral reservoir establishment, we purified two subsets of CD4 T cells that express different levels of SAMHD1, namely germinal center enriched Tfh, defined as CXCR5^{+}ICOS^{bright}PD-1^{bright}, and quiescent triple negative (TN), defined as CXCR5^{neg}ICOS^{neg}PD-1^{neg} as depicted in Figure 5.

These cells have distinct levels of SAMHD1(Passos et al., 2022) which we have also observed (Figure 5): Tfh presented high levels of Bcl-6, the mentioned Tfh master transcription regulator, and, consequently, lower levels of SAMHD1, whereas the quiescent TN have consistently higher levels of SAMHD1, both transcriptionally (Figure 6A) and at a protein expression level (Figure 6B). Interestingly, TN homogeneously express high levels of SAMHD1 protein, while SAMHD1 expression in germinal center enriched Tfh was heterogeneous (Figure 6B).

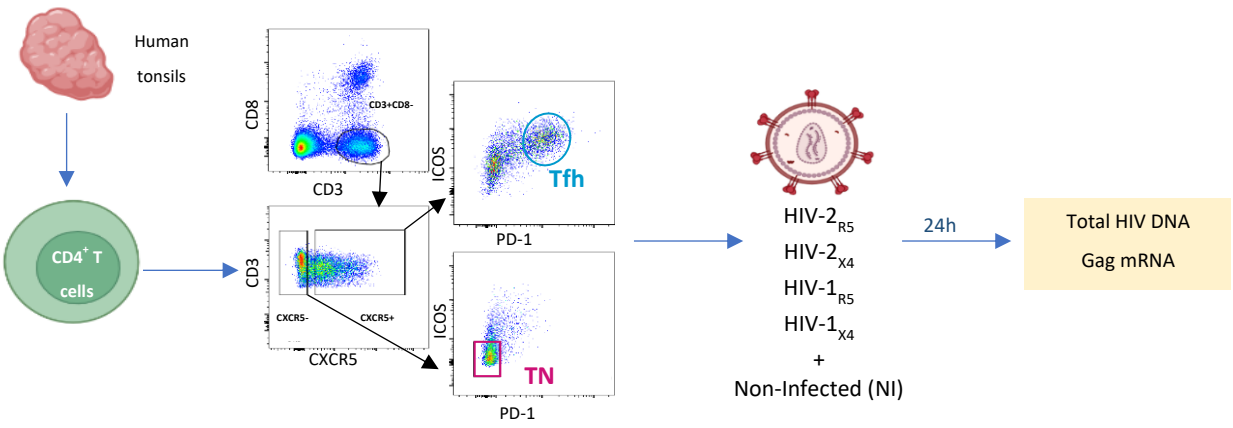

Figure 5. Experiment outline illustrating the isolation from human tonsils of germinal center enriched follicular helper cells (Tfh - CXCR5^{+}ICOS^{bright}PD-1^{bright}) and in quiescent triple negative (TN - CXCR5^{neg}ICOS^{neg}PD-1^{neg}) CD4 T cells, and analysis 24h upon infection with HIV-2 and HIV-1 primary isolates defined according to CCR5 or CXCR4 usage.

We compared their ability to support infection by HIV-2 and HIV-1 primary isolates, using either CCR5 or CXCR4 as coreceptor, as outlined in Figure 5. Significantly higher

levels of total HIV DNA were found in Tfh as compared to TN, for all the viral isolates, upon 24h infection (Figure 6C), in agreement with the recognized high ability of Tfh to support viral reservoir establishment (Godinho-Santos et al., 2020; Perreau et al. 2013).

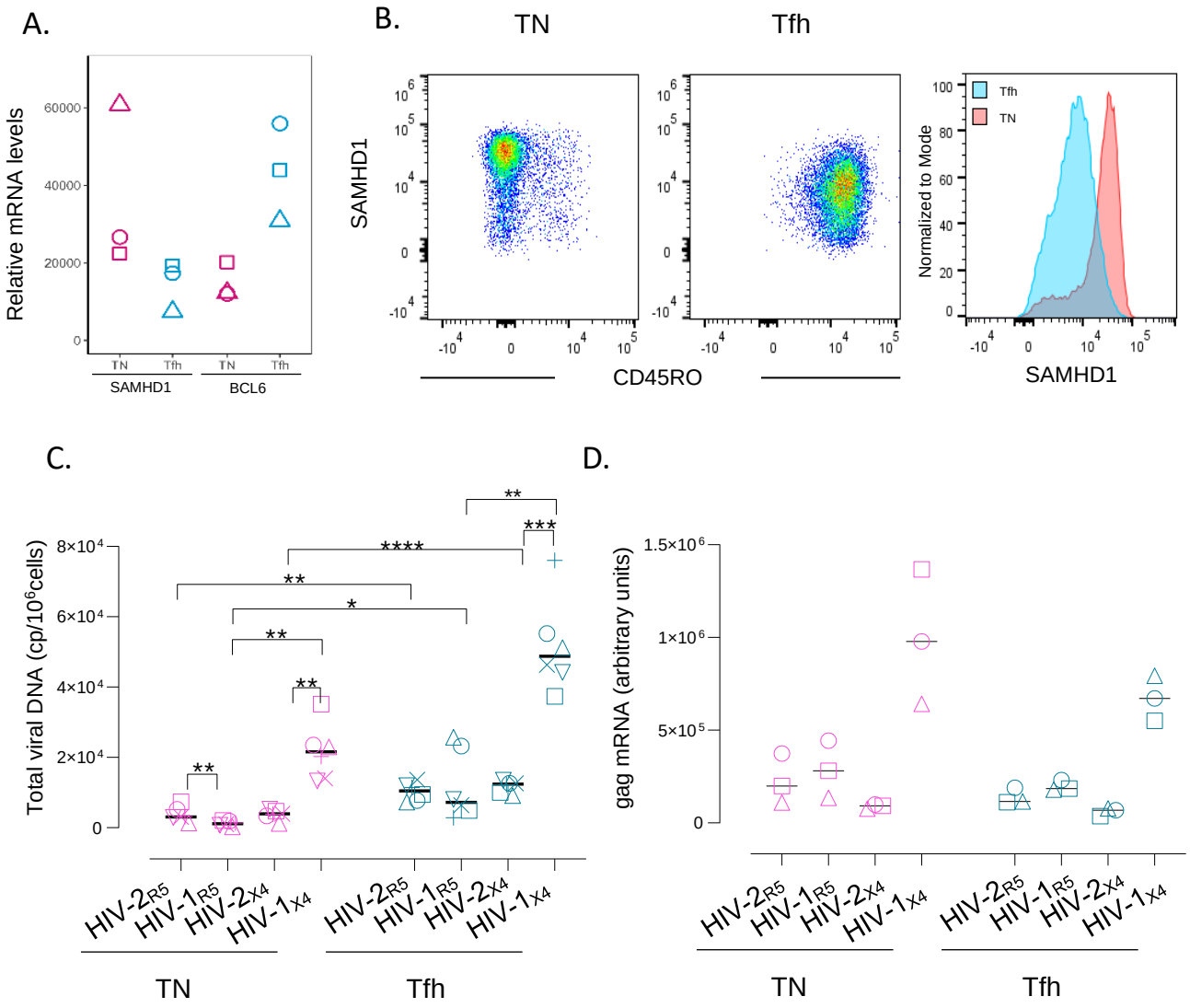

Figure 6. *Analysis of Tfh and TN from human tonsils before and after infection.* A) *SAMHD1* and *BCL6* expression in non-infected follicular helper T cells (Tfh – blue – $CD3^{+}CD4^{+}CXCR5^{+}ICOS^{bright}PD\text{-}1^{bright}$) as compared to non-infected quiescent triple negative (TN – red – $CD3^{+}CD4^{+}CXCR5^{neg}ICOS^{neg}PD\text{-}1^{neg}$) purified tonsillar CD4 T cells after 24h culture; each symbol corresponds to a donor. B) SAMHD1 protein levels analyzed by flow cytometry in sort-purified TN and Tfh from an illustrative human tonsil; dot-plots on the left show SAMHD1 levels according to CD45RO expression, and on the right the overlay histogram compares SAMHD1 levels in TN and Tfh. C and D) Quantification total cell-associated viral DNA (C) and viral *gag* mRNA levels (D) in Tfh and TN populations; each symbol corresponds to a donor tonsil; data were compared using one-way Anova ($p<0.0001$) and Benjamini Krieger and Yekutieli test with significant p values shown as ****$p<0.0001$, ***$p<0.001$, **$p<0.01$, *$p<0.05$.

Upon comparison of the levels of HIV-2 and HIV-1 total DNA in the TN samples, we found opposite effects according to coreceptor usage, namely significantly higher levels in HIV-2_{R5} than HIV-1_{R5} (p=0.0071) while the levels were lower in HIV-2_{X4} than in HIV-1_{X4} (p=0.0032)(Figure 6C). Notably, in Tfh, there was no major impact of coreceptor tropism in the case of HIV-2, in contrast with HIV-1 where the CXCR4-using virus was associated with significantly higher levels than the CCR5-using HIV-1 of total DNA (Figure 6C). Moreover, the levels of viral mRNA were not significantly higher in HIV-2 for both CCR5- and CXCR4-using isolates as compared to their HIV-1 counterparts, upon infection of either TN or Tfh (Figure 6D).

These findings suggest that the levels of SAMHD1 have reduced impact on the CD4 T cell ability to support HIV-2 infection in contrast to HIV-1. Because SAMHD1 expression in germinal center enriched Tfh was heterogeneous (Figure 6B), we investigated SAMHD1 expression within Tfh and its putative overlap with several markers distinctive of Tfh by applying an unsupervised approach (UMAP) (Figure 7).

Tfh were analyzed after dead cells, debris and doublets were excluded, as shown in Figure 7A. Tfh cells were defined as previously shown (Figure 5) within CD45+ cells. As expected, Tfh phenotype is marked by the expression Bcl-6, the master transcription regulator for the differentiation of Tfh. These specialized activated cells also express CD45RO as expected since it is associated with memory CD4 T cells. We also observed high levels of CXCR4, the beforementioned HIV coreceptor, and lower levels of CCR5, the other coreceptor that still presents to some degree of expression. During their progression towards the germinal center, these cells upregulate markers such CD69, maturation marker CD57, and OX40, the T cell costimulatory receptor. As illustrated in Figure 7B, there was poor overlap between the patterns of expression of SAMHD1 and main germinal center markers, such as Bcl-6, CXCR4, OX40, and CD57.

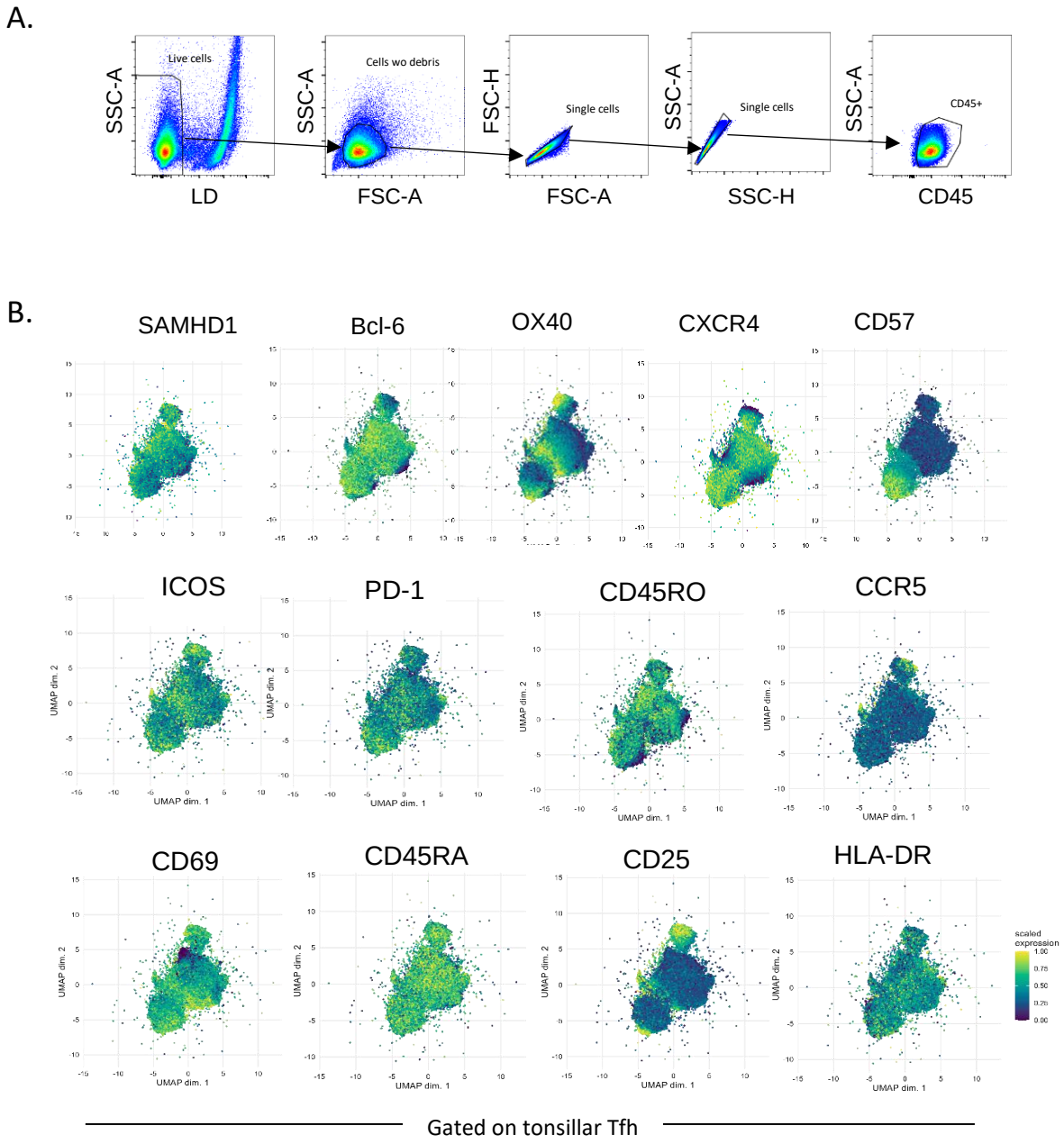

Figure 7. SAMHD1 expression in Tfh. A) Illustrative flow cytometry gating strategy. B) Detailed phenotypical ex-vivo analysis of Tfh ($CD3^{+}CD4^{+}CXCR5^{+}ICOS^{bright}PD\text{-}1^{bright}$) using UMAP, concatenated data from 9 human tonsils are shown.

Tfh differentiation from naïve cells infected with HIV-2 or HIV-1

Next, we hypothesized whether HIV-2 Vpx-mediated SAMHD1 downmodulation may impact on Tfh differentiation, given the interplay of SAMHD1 and Bcl-6 in this process(Cai et al., 2019). To investigate this possibility, we compared the impact of the presence of HIV-2 and HIV-1 using an *in vitro* assay of Tfh differentiation, previously optimized by the group of Ueno(Schmitt et al., 2014). Naïve CD4 T cells were sort-

purified from the peripheral blood of healthy donors, and infected with HIV-2 and HIV-1 primary isolates as outlined in Figure 8.

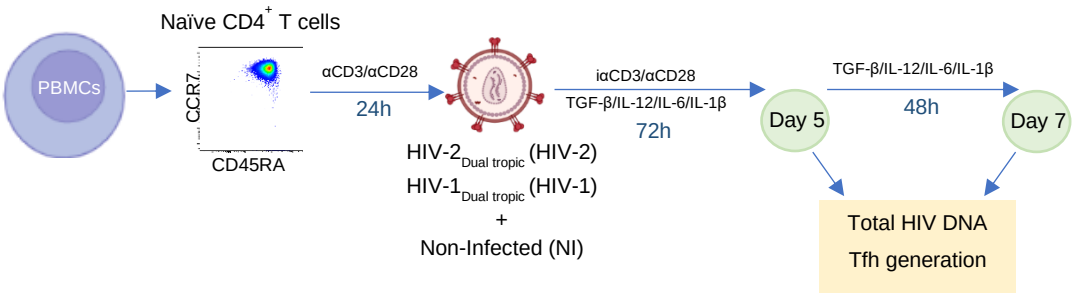

Figure 8. Experimental design illustrating the naïve CD4 T cell isolation from peripheral blood, their infection using dual-tropic HIV-2 and HIV-1 primary isolates, and stimulation protocol for Tfh differentiation.

We selected dual-tropic HIV-2 and HIV-1 viruses to circumvent the impact of coreceptor usage and found that the levels of cell-associated total DNA were comparable at 72h post-infection, mentioned as day 5, between HIV-1 and HIV-2 (Figure 9A). We observed an increase in total HIV DNA from day 5 to day 7 for all donors and both viral isolates. Notably, this increase is less pronounced in HIV-2 in contrast to HIV-1.

We also quantified the frequency of CXCR5+ cells to globally estimate Tfh in the culture at day 5 and day 7, after excluding dead cells, debris, and doublets, as previously displayed in Figure 7A. As shown in figure 9B, there was an elevation in the percentage of CXCR5+ cells from day 5 to day 7, corresponding to Tfh expansion. When comparing the three conditions, we note that there is no correlation between infection conditions and Tfh expansion.

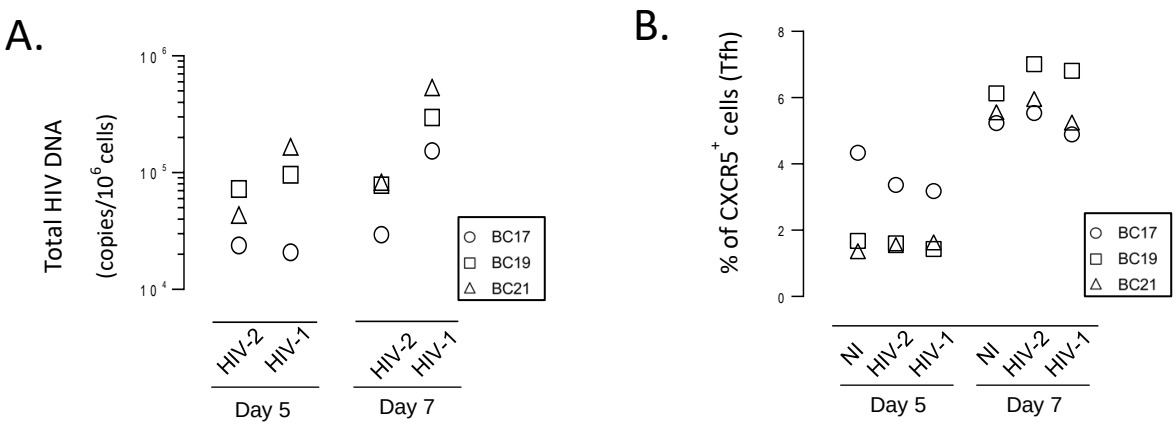

Figure 9. A) Quantification of total cell-associated viral DNA at day 5 and day 7 in HIV-2 and HIV-1 infected samples; each symbol represents a donor. B) Frequency of CXCR5+ cells at day 5 and day 7 in non-infected (NI) and infected by HIV-2 or HIV-1 in the same donors as in (A).

Additionally, we applied an unsupervised approach (UMAP) to analyze the kinetics of expression of a panel of markers associated with Tfh function (Bcl-6, PD-1, ICOS, OX40, CD57) and general markers of cell activation/differentiation (CD45RA/RO, CD69, CD25, Foxp3, CCR6, CXCR3) along the culture. As shown in Figure 10, the main determinant of the UMAPs was the donor of the naïve cells and the evolution patterns were clearly similar between the infection conditions.

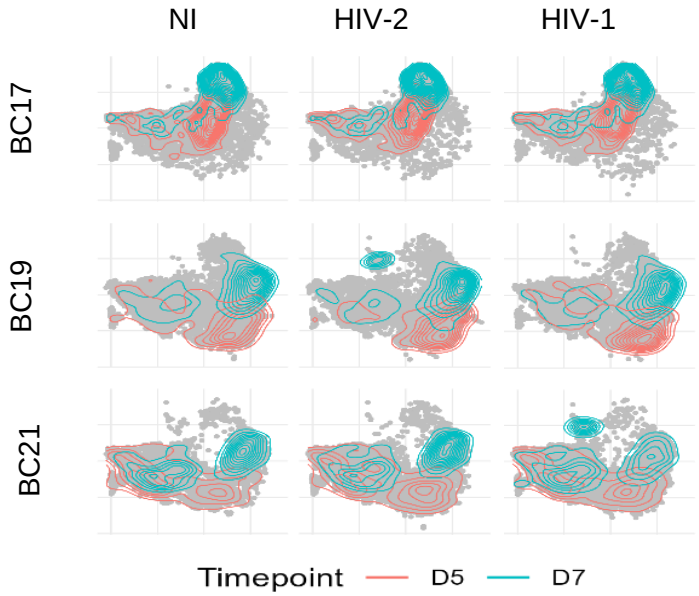

Figure 10. Comparison of cell differentiation at day 5 (red) and day 7 (blue), in the different donors and conditions; unsupervised analysis of flow cytometry data using UMAP

We then went to perform a phenotypic characterization of the generated CXCR5+ cells comparing the different infection conditions (Figure 11). We observed a lack of significant impact of HIV-2 infection, where a remarkable similarity can be noticed when comparing all three conditions for each marker. Thus, phenotype does not seem to change significantly upon infection with either virus. Just as described in tonsillar Tfh, the cells exhibit a heterogeneous expression of SAMHD1, while expressing different levels of Bcl-6, PD-1 and ICOS. Although CD45RO is highly expressed in all conditions, there are some cells that do not express this marker. The generated cells featured some expression of GC markers OX40 and CD57, although in a lower scale for CD57. Interestingly, there is a small population with high levels of CD57.

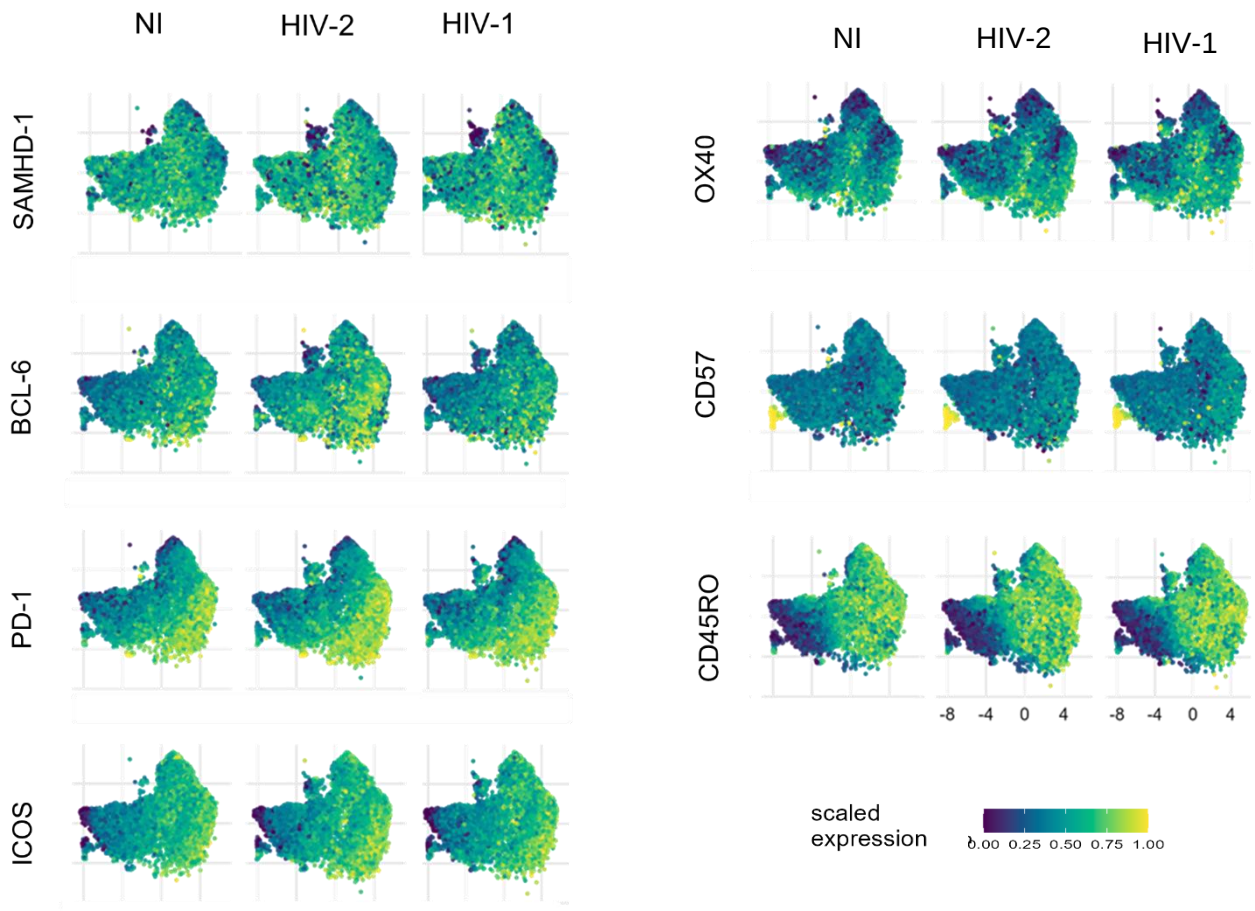

Figure 11. Phenotypical analysis of Tfh (CXCR5+) at day 7 using UMAP, concatenated data from the 3 donors in each condition are shown.

Altogether, these first data on the impact of HIV-2 infection on *in vitro* Tfh differentiation did not reveal major alterations both in the yields and the final phenotype of the generated CXCR5+ cells, this supports an absence of any impact of SAMHD1 downmodulation through HIV-2 in Tfh differentiation.

Discussion

In this study we investigated the interplay of SAMHD1 and HIV in Tfh, a major HIV-1 and HIV-2 reservoir (Godinho-Santos et al., 2020; Perreau et al., 2013), by comparing the ability of reservoir establishment in two CD4 T cell subsets with distinct SAMHD1 expression levels, Tfh and TN. Both TN and Tfh cells were able to support infection by both HIV-1 and HIV-2, as shown in figure 6. Higher levels of total HIV DNA were found in Tfh when compared to the quiescent TN, reinforcing the ability of Tfh to establish viral reservoirs. Interestingly, the differences between Tfh and TN were more evident in HIV-1 than HIV-2. Previous studies have demonstrated an enhancement of HIV-1 infection through virion packaged Vpx from SIV in resting CD4 T cells (Baldauf et al., 2017), which could lead to the assumption that HIV-2 Vpx could also increase viral infection in T cells. Even though studies have shown that low SAMHD1 levels correlate with a crucial role in viral susceptibility, with Tfh cells presenting notably high levels of viral replication, data regarding the modulation of this restriction factor is scarce (Ruffin et al., 2015). Moreover, many factors, such as immune activation and IFN induction, impact SAMHD1 expression and activity and henceforth affect its ability to impair HIV replication (Li et al., 2020). We did not evaluate the activation state of TN neither did we assess SAMHD1 expression upon infection to correlate with these possible factors, which could give more insights for our results showing successful infection in both TN and Tfh populations. Despite the described degradation of SAMHD1 through HIV-2 Vpx, other strategies might be necessary to understand SAMHD1 activity and its definitive effect in cell susceptibility in different subsets and activation status.

This study also aimed to investigate the impact of HIV-2 infection, and consequently SAMHD1 downmodulation through Vpx, on the differentiation of Tfh. In this *in vitro* assay, we found no evidence supporting a direct impact of SAMHD1 downmodulation through HIV-2 on Tfh differentiation as neither the quantity nor the quality of generated Tfh seemed to be affected. In fact, the frequency of Tfh was hardly different between conditions at days 5 and 7, as well as the cell phenotype which was also similar through all conditions for the different markers. Nevertheless, we cannot exclude the possibility

that the low frequency of generated CXCR5+ cells might mask some evidence. Another possible factor might be that Vpx from HIV-2 could not reduce sufficiently SAMHD1 expression in naïve CD4 T cells to promote an impact on Tfh differentiation. Indeed, it has been demonstrated that the efficient downregulation on SAMHD1 is achieved through Vpx transfection (Mohamed et al., 2021). Of note, studies have found that although HIV-2 RT and HIV-1 RT produce relatively identical results in quantitative functional studies, when under conditions of limiting RT concentrations, HIV-1 RT is substantially more effective (Post et al., 2003). Also, it is possible that Vpx may have underexplained other possible targets to exert additional effects within host cells, such as inhibition of cGAS–STING-triggered NF-κB signalling (Su et al., 2019). Finally, SAMHD1 affects cell metabolism, proliferation, and immune responses; thus, it is critical to take these multiple functions upon consideration for comprehending the effects of SAMHD1 in HIV infection and cellular biology.

For further studies it will be important to perform single cell analysis of CD4 T cells infected by HIV-2 that may reveal new perspectives on the role of Vpx and SAMHD1 counteraction. Given its role in cell biology, namely in cellular DNA replication, SAMHD1 knockdown is difficult to achieve which limits experimental design in current studies. Another important aspect that we did not address is to consider the analysis of SAMHD1 localization in the cell and its trajectory to and from the nucleus as well as its phosphorylation status.

It is also rather difficult to sort well-defined Tfh subsets based on SAMHD1 expression given its heterogeneous expression pattern and lack of sufficient overlay with other cell markers, which, if possible, could allow a more detailed analysis of SAMHD1 impact in these viral reservoirs.

Further optimization of the Tfh differentiation protocol is required to reach higher frequencies of generated CXCR5+ cells in order to produce even more significant data as well as perform significant statistical tests.

Conclusion

In conclusion, this study provides novel insights into the infection of CD4 T cell subsets with HIV-2 and the impact of HIV-2 infection on Tfh differentiation. We observed higher total HIV DNA levels in Tfh when compared to TN, accordingly to their ability to support infection and serve as viral reservoirs. Surprisingly, the differences between Tfh and TN were more pronounced in HIV-1, contrary to the expectation that HIV-2, harbouring Vpx, should have some sort of advantage in the infection of these cells. Additionally, this study addressed the impact of HIV-2 infection in Tfh differentiation using an *in vitro* model with no significant changes in either the quantity or quality of the generated Tfh. These first data using HIV-2 primary isolates will prompt the investigation of additional paths in the establishment of Tfh reservoirs, and their implications for the benign course of HIV-2.

Authors contributions

André Pires performed tonsil processing and TMNC phenotyping by flow cytometry acquisition, as well data processing and analysis. André Pires assisted in immunophenotyping staining and analysis during the *in vitro* differentiation protocol which was mainly performed by Ana Godinho-Santos, Carolina Conceição and Rita Moura in BSL3. CD4 T cell subset infection and collection of viral and cellular transcripts was performed by Ana Godinho-Santos, Ana Antão, Bárbara Tavares, and Tiago Ferreira in 2017 and 2019. André Pires assisted in the amplification and qPCR optimizations of SAMHD1 and Bcl-6 transcripts, in collaboration with Guilherme Farias who also participated in data analysis.

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Communications

The data in this work was previously presented by the author in the form of poster in "Dia da Investigação" in Faculdade de Medicina da Universidade de Lisboa as a conclusion of the GAPIC project, in 2021 and 2022. It was also presented in the *Sociedade Portuguesa de Imunologia* meeting in 2021 by Rita Moura, on-line, and in 2023, by the author, in the Aveiro Conference Center, in Aveiro, Portugal; as well as in the AIMS Meeting Research Competition 2023 which happened online.

The work was also presented in poster format by Robert Badura, MD, in the Conference on Retroviruses and Opportunistic Diseases (CROI 2023), in the Summit Convention Center in Seattle, Washington, USA.

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