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Ana Raquel Lourenço de Sousa

Licenciatura em Bioquímica

HIV-1 infection on Follicular Helper T cells

Dissertação para obtenção do Grau de Mestre em
Bioquímica para a Saúde

Orientador: Doutora Helena Soares, Principal Investigator,
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Resumo

A infecção pelo Vírus da Imunodeficiência Humana (VIH) é considerada uma doença controlável sob terapia anti-retroviral, embora ainda não seja capaz de erradicar os reservatórios celulares que sustentam a contínua replicação de VIH-1. As células T auxiliares do tipo folicular (T_{FH}) foram indicadas como o maior santuário de VIH-1. Estas células localizam-se principalmente nos centros germinativos (CGs), dos orgãos linfáticos secundários, e são especializadas em induzir a produção de anticorpos pelos linfócitos B. Apesar do seu papel crucial na infecção pelo VIH-1, ainda existem muitas lacunas em relação ao modo como o VIH-1 explora a sua maquineria celular de forma a proliferar. Por isso, o objectivo deste trabalho é contribuir para elucidação deste mistério através do isolamento de linfócitos $CD4^+$ de amígdalas de dadores humanos saudáveis, infectando-os com VIH-1 *ex vivo* e identificando a população de T_{FH} por citometria de fluxo.

Este trabalho apresenta evidências em como o VIH-1 preferencialmente infecta e expande a população de CG T_{FH} através do aumento da expressão do seu factor de transcrição Bcl6 e receptor quimiotáctico CXCR5, também aumenta a sua sobrevivência e proliferação, medida pela proteína nuclear Ki67. O VIH-1 impõe um estado de activação (expressão de CD69 aumentada) e, consequentemente, um metabolismo acrescido, especificamente a via metabólica fosforilação oxidativa (massa mitocondrial aumentada), para fazer face às exigências energéticas da replicação viral e manutenção do reservatório celular. Adicionalmente, este vírus modula a sinalização das T_{FH} através do aumento da expressão do receptor de células T-CD3, receptor do tipo toll 7 e citoquinas pró-inflamatórias (IL-21, IL-17 e IFNγ) de forma a promover a sua sobrevivência e proliferação.

Estes resultados constituem o trabalho de base fundamental e propiciam avenidas de investigação promissoras para elucidar o mecanismo de infecção pelo VIH-1 neste reservatório celular visando o desenvolvimento de estratégias de curas funcionais.

Palavras-chave: VIH-1, células T auxiliadoras do tipo folicular, reservatório celular, sinalização celular

Abstract

Human Immunodeficiency Virus (HIV) infection is considered a manageable disease under antiretroviral therapy, although it is still unable to eradicate cellular reservoirs that harbor ongoing HIV-1 replication. Follicular helper T (T_{FH}) cells were pointed as the major HIV-1 sanctuary. They are mainly located in germinal centers (GCs), within secondary lymphoid organs, and are specialized in inducing antibody production from B cells. Despite their crucial role in HIV-1 infection, there is still a lot of gaps to fill regarding how this virus exploits their machinery in order to thrive. For that reason, the aim of this work is to take a step closer to unveil this mystery by isolating $CD4^+$ T cells from healthy human tonsils, infect them *ex vivo* with HIV-1 and identify the T_{FH} population by flow cytometry.

This work presents evidences that HIV-1 preferentially infects and expands GC T_{FH} population by upregulating its transcription factor Bcl6 and chemokine receptor CXCR5, it also increases its survival and proliferation, measured by nuclear protein Ki67. HIV-1 imposes a state of activation (increased CD69) and, consequently, augmented metabolism, specifically the oxidative phosphorylation metabolic pathway (increased mitochondrial mass) to meet it energy demands for replication and cellular reservoir maintenance. Furthermore, this virus modulates T_{FH} signaling by upregulating T-cell receptor-CD3, Toll-like receptor 7 and pro-inflammatory cytokines (IL-21, IL-17 and IFNγ) to promote its survival and proliferation.

Overall, these findings reveal the urgent need to investigate HIV-1 infection mechanism in this cellular reservoir aiming at developing functional cure strategies.

Keywords: HIV-1, Follicular helper T cells, cellular reservoir, cell signaling

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Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
APCs	Antigen-presenting cells
ART	Antiretroviral therapy
Bcl6	B-cell lymphoma 6 protein
BSA	Bovine serum albumin
CCL	Chemokine (motif C-C) ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CXCL	Chemokine (C-X-C motif) ligand
CXCR	C-X-C chemokine receptor
DCs	Dendritic cells
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
EIs	Entry inhibitors
FACS	Fluorescence-activated cell sorting
FACS-SAP	Fluorescence-activated cell sorting-Saponin
FBS	Fetal bovine serum
FDCs	Follicular dendritic cells
FIs	Fusion inhibitors
FSC	Forward Scatter
GCs	Germinal centers
GFP	Green fluorescent protein
HBS	Hepes-buffered saline
HIV	Human Immunodeficiency Virus
ICOS	Inducible T-cell co-stimulator
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
iMM	Instituto de Medicina Molecular

IMQ	Imiquimod
INs	Integrase inhibitors
L/D	Live/Dead cells
LB	Luria-Bertani
MFI	Mean fluorescence intensity
MHC	Major Histocompatibility
MNCs	Mononuclear cells
mRNA	Messenger RNA
MT	Mitotracker
NF-ƙB	Nuclear factor-kappa B
nGC	Non-Germinal center
NKs	Natural killer cells
NNRTIs	Non-nucleoside reverse transcriptase inhibitors
NRTIs	Nucleoside/Nucleotide reverse transcriptase inhibitors
nT_{FH}	Non-T_{FH}
O/N	Overnight
P/S	Penicillin-Spreptomycin
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PD-1	Programmed cell death protein 1
PFA	Paraformaldehyde
PIs	Protease inhibitors
PLL	Polylysine
PRRs	Pattern recognition receptors
RNA	Ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SIV	Simian Immunodeficiency Virus
SSC	Side Scatter
ssRNA	Single-stranded RNA
TCR	T-cell receptor

T_{FH}	Follicular helper T cells
Th	T helper cells
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
WHO	World Health Organization

1. Introduction

1.1. Human Immunodeficiency Virus (HIV)

1.1.1 History

AIDS (Acquired Immunodeficiency Syndrome) is characterized by a progressive failure of immune system leading to opportunistic infections and it is transmitted by bodily fluids as semen or blood (Fanales-Belasio, E. [et al.], 2010). It was first clinically described in the USA, in 1981, in a restrict group of homosexual men, intravenous drug users and hemophiliacs (Sharp, P.M. and Hahn, B.H., 2011). However, the number of cases continued to grow and it was not confined to the previously described community (Greene, W.C., 2007). In 1983-84, two separate research groups, led by Luc Montagnier and Françoise Barré-Sinoussi (Barresinoussi, F. [et al.], 1983) and Robert Gallo (Gallo, R.C. [et al.], 1983), identified HIV (Human Immunodeficiency Virus) as the cause of this pandemic disease (figure 1.1).

Later on, it was discovered the existence of two closely related viruses: HIV-1 and HIV-2. Both of them are believed to be originated from non-human primates in southern Cameroon infected with Simian Immunodeficiency Viruses (SIVs), by hunting or handling bushmeat (zoonosis). Due to rubber and ivory exploitation, there were fluvial connections between Cameroon and Kinshasa (capital of Democratic Republic of Congo), allowing the virus to travel into this growing city, in 1920. Being the biggest city in the region with a rapid population growth, sex trade, railways and emigration, offered the virus conditions to begin spreading from human-to-human regionally, at first, and soon after around the world (Faria, N.R. [et al.], 2014). In fact, it is believed that HIV entered in the USA, via New York City, from Caribbean (Worobey, M. [et al.], 2016).

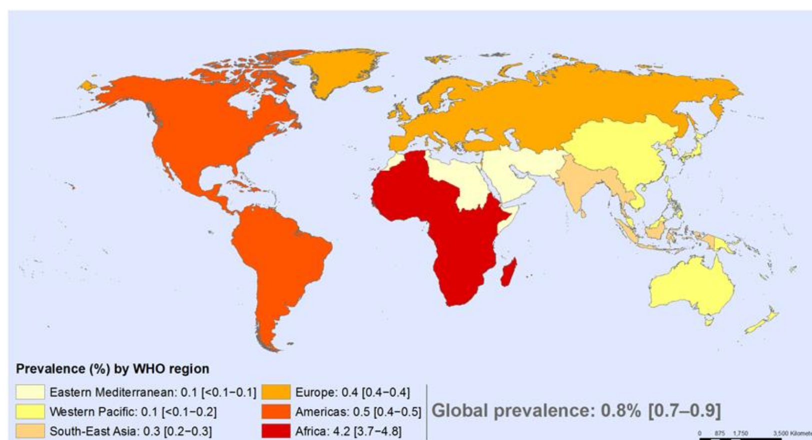

Figure 1.1 - Worldwide Human Immunodeficiency Virus (HIV) prevalence in adults aged 15 to 49, in 2016. *From World Health Organization (WHO).*

HIV-1 is the worldwide main AIDS agent since it is more virulent and has a higher transmission rate, while HIV-2 is restricted to Western and Central Africa. Moreover, HIV-1 can be divided into four groups: M, N, O and P. HIV-1 group M is the most common strain and within it are known nine subtypes (A, B, C, D, F, G, H, J and K), whereas the other three groups are a minority. Whilst HIV-1 groups N, O and P are usually confined to West-Central Africa, the M group is worldwide spread (Taylor, B.S. and Hammer, S.M., 2008). Interestingly, Portugal is one of the countries with the major HIV-1 subtype's diversity as B (27%), G (29.1%), C (14.5%) and recombinant forms (17.6%), among other subtypes, which is currently a matter of study (Carvalho, A. [et al.], 2015).

1.1.2 Structure and genome

HIV-1 is a lentivirus, a subgroup of retrovirus, which is composed by two copies of single-stranded ribonucleic acid (ssRNA) and it is characterized by long periods of incubation (figure 1.2). Its genome consists in three structural genes (*gag*, *pol* and *env*) and six regulatory genes (*tat*, *rev*, *nef*, *vif*, *vpr* and *vpu*). The *gag* gene encodes the precursor gag polyprotein which is processed upon viral maturation in structural core proteins (p24, p7 and p6) and matrix protein (p17). The *pol* gene codes for the enzymes required for viral replication: reverse transcriptase, RNase H, integrase and protease. The *env* gene encodes the precursor envelope protein gp160 which is cleaved in gp120 and

gp41 (Fanales-Belasio, E. [et al.], 2010). The regulatory genes are also important in modulating virus replication: increasing gene transcription (Tat protein), transport of viral mRNA (messenger RNA) from the nucleus to the cytoplasm (Rev protein) and manipulation of host machinery to establish a permanent state of infection (Nef protein – virulence factor) (Emerman, M. and Malim, M.H., 1998).

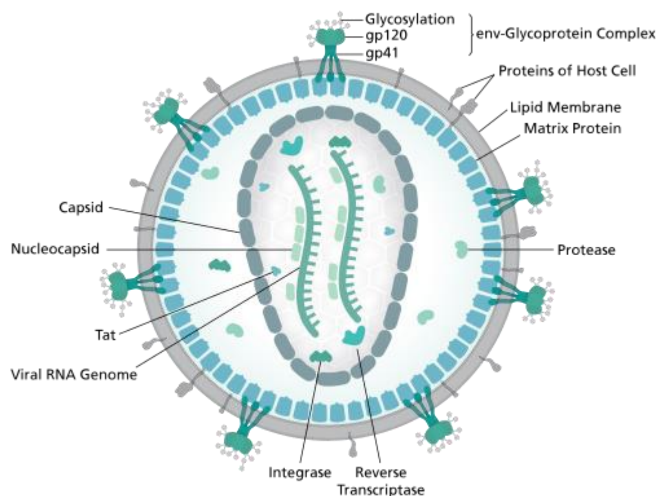

Figure 1.2 - Structure of HIV. *From Thomas Splettstoesser (www.scistyle.com).*

1.1.3 Replication cycle

HIV-1 specifically infects immune cells that express the surface CD4 receptor (cluster of differentiation 4), such as $CD4^+$ helper T cells, macrophages and dendritic cells (DCs). Considering that the first ones are $CD4^{high}$, they are HIV main target cells; while macrophages and DCs are $CD4^{intermediate}$, meaning that they are less extensively infected (Wilen, C.B. [et al.], 2012). HIV also recognizes the C-C chemokine receptor type 5 (CCR5) - R5 viruses, C-X-C chemokine receptor type 4 (CXCR4) - X4 viruses or both CCR5 and CXCR4 - R5X4 viruses (Berger, E.A. [et al.], 1998).

The infection specificity it is related with HIV entry pathway into the host cell (figure 1.3), since it requires the interaction between its envelope glycoproteins (gp120 and gp41) and surface receptors on the target cell (Wyatt, R. and Sodroski, J., 1998). Gp120 first interacts with CD4 molecule which induces a conformational change that allows a secondary interaction with the co-receptor(s), thus gp41 can be inserted into the cellular membrane initiating membrane fusion and consequently viral capsid entry – fusion and entry stage (Fanales-Belasio, E. [et al.], 2010).

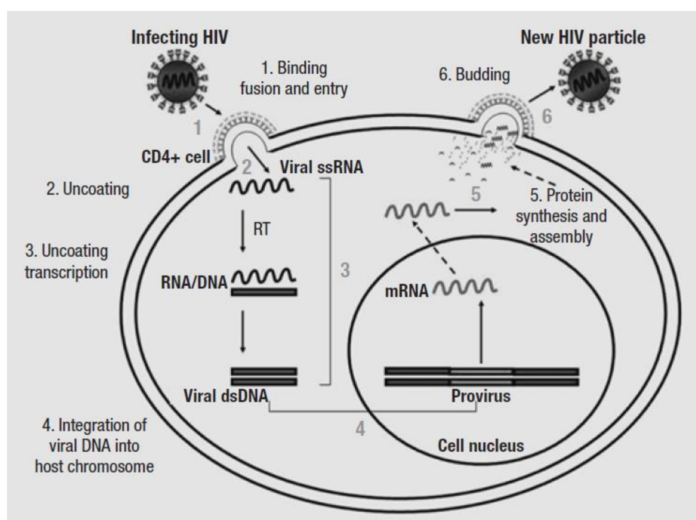

Figure 1.3 - HIV life cycle. *From (Fanales-Belasio, E. [et al.], 2010)*

After that, the viral RNA is released into the host cytoplasm (uncoating process) and it is transcribed into proviral DNA (Deoxyribonucleic acid), by reverse transcriptase and RNase H. Then, it is integrated into the host genome by integrase, where it may lay in a latent state – reverse transcription and viral DNA integration stage. Viral replication requires that the host immune cell is in an activated state, since important host transcription factors (as nuclear factor-kappa B (NF-ƙB)) are required for HIV transcription (Wu, Y.T. and Marsh, J.W., 2003). This explains the cell depletion firstly in the gut mucosa, leading to a microbial translocation from the gut to the blood which is thought to have a role in further driving inflammatory activation (Tincati, C. [et al.], 2016).

The next stage on HIV replication cycle is the viral gene expression which begins with the transcription of proviral DNA into viral mRNA, in the host nucleus. It migrates into the host cytoplasm where it is translated into viral proteins that will form the new viral particles (Wu, Y.T. and Marsh, J.W., 2003). Afterward there is viral assembly, at the host cellular membrane, mediated by Gag polyprotein, wherein all essential components are packaged. Finally, the new viral particle is released from the host cell (budding process) in which it acquires its envelope (enriched in host phospholipids). After its release, it undergoes a maturation process resulting in a virion – a mature infectious viral particle (Sundquist, W.I. and Krausslich, H.G., 2012).

1.1.4 Pathogenicity

There are three stages of HIV infection: acute, chronic and AIDS (figure 1.4). The acute stage usually lasts for 2-4 weeks and, on the first 10 days (eclipse phase), the virus is not detectable in the plasma. During this phase, mucosal $CD4^+$ T cells, macrophages and DCs are infected, breaching the mucosal barrier and allowing HIV penetration and spread (Perreau, M. [et al.], 2013a). At this point, the virus is detectable in the plasma and there is an exponential infection increase, where there is a decline of $CD4^+$ T cell count, characterized by flu-like symptoms (Kahn, J.O. and Walker, B.D., 1998).

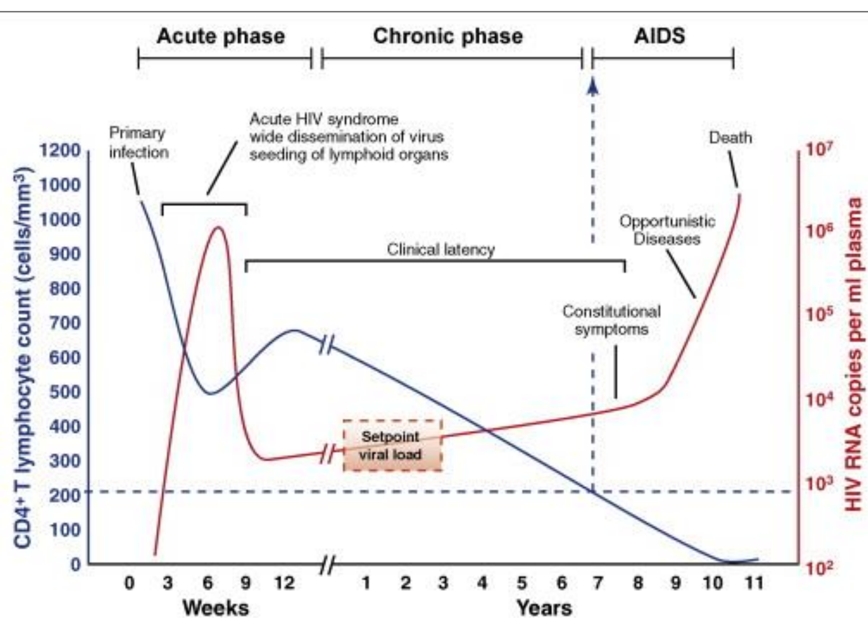

Figure 1.4 - HIV course of infection. *From (An, P. and Winkler, C.A., 2010)*

The infection is amplified once infected cells are drained to lymph nodes, where they encounter activated T cells and a large production of pro-inflammatory cytokines, as interleukin-6 (IL-6) and tumor necrosis factor α (TNF-α) (Katsikis, P.D. [et al.], 2011). Thus, the perfect conditions for rapid HIV replication are established (Perreau, M. [et al.], 2013a). Meanwhile, the immune system is trying to fight the infection by activating $CD8^+$ T cells and B cells to begin to secrete HIV-specific antibodies. $CD8^+$ T cells recognize infected cells through viral antigen displayed by class I Major Histocompatibility (MHC-I) and secrete perforin and granzymes to lyse infected cells (Gulzar, N. and Copeland, K.F.T., 2004). This is the most important immune response in controlling HIV infection, by the end of the acute stage, leading to a decrease and stabilization of the viral titer -

viral set point. The other component of HIV-immune response is the seroconversion, which begins in the acute phase but it extends to the chronic phase. The seroconversion is a process where plasma cells (mature B cells) secrete HIV-specific antibodies (Hecht, F.M. [et al.], 2011).

The chronic stage (asymptomatic period) is characterized by a clinical latency due to low viral load and it can last for 10 years or more. Despite all the efforts taken by the immune system, infected cells became dysfunctional. Consequently, their responses are insufficient to restrain the infection (lack of effective $CD8^+$ response and inadequate $CD4^+$ T cell help leads to poor antibodies responses), inducing a state of chronic infection (McMichael, A.J. [et al.], 2010). This chronic state of immune activation also damages its responses, increasing the progressive depletion of $CD4^+$ T cells (< 200 cells/µL of plasma) and the proliferation of HIV (Fanales-Belasio, E. [et al.], 2010). Therefore, after years of consistent destruction of the immune system, it culminates with totally loss of immune responses, leaving the body extremely susceptible to opportunistic infections, known as AIDS (Brooks, J.T. [et al.], 2009).

1.1.5 Antiretroviral Therapy

According to the World Health Organization (WHO), more than 70 million people have been infected by HIV and about 35 million people died from it (http://www.who.int/gho/hiv/en/), so finding a way to control or even to totally suppress HIV infection is still a main concern of nowadays society. So far, it was developed six classes of antiretroviral drugs that act on different stages of HIV replication cycle and are usually prescribed in combination:

- Entry inhibitors (EIs): target the co-receptor CCR5, interfering with HIV bidding and entry into the host cell (e.g. Maraviroc);
- Fusion inhibitors (FIs): disrupt HIV-target cell fusion by mimicking the components of HIV fusion machinery (e.g. Enfuvirtide);

- Nucleoside/Nucleotide reverse transcriptase inhibitors (NRTIs): nucleoside/nucleotide analogues that inhibit reverse transcription, by acting as competitive substrate inhibitors (e.g. Zidovine);
- Non-nucleoside reverse transcriptase inhibitors (NNRTIs): interfere with reverse transcription by acting as non-competitive substrate inhibitors (e.g. Rilpiverine);
- Integrase inhibitors (INs): impair proviral DNA integration into the host genome (e.g. Raltegravir);
- Protease inhibitors (PIs): block viral protease, preventing cleavage of Gag/Pol precursors proteins during viral maturation, leading to the production of defective viral particles (e.g. Amprenavir) (Arts, E.J. and Hazuda, D.J., 2012).

The major challenge imposed by HIV is its genetic variability which counteracts both effective antiretroviral therapy (ART) and host immunity. This variability is interconnected with HIV rapid replication (around 10^{10} virions/day), recombination of different viruses within the same infected individual and high mutation rate by the action of reverse transcriptase (around 0.2 errors/genome/cycle) (Rambaut, A. [et al.], 2004).

1.1.6 Traditional HIV reservoirs

Furthermore, there is another serious obstacle to HIV eradication: latent HIV reservoirs. They act as sanctuaries since they harbor HIV without actively producing it, meaning that these group of immune cells are in a resting state and, for that reason, they are not accessible to antiretroviral drugs. Approximately two weeks upon ART discontinuation, there is viral rebound, meaning that infected individuals require life-long therapy which obviously entails several concerns (virus resistance, cumulative toxicities and economic concerns) (Chun, T.W. [et al.], 2015).

These reservoirs are constituted by resting memory $CD4^+$ T cells, macrophages and DCs and they are established during the acute phase of infection course, which is why is so important to initiate ART as early as possible. Memory $CD4^+$ T cells are characterized for their long life span and quiescent state, maintaining basic cellular processes in order to survive and to respond upon immunologic stimuli (Alexaki, A. [et al.], 2008). Although

circulating memory $CD4^+$ T cells have been described as the hotspot of HIV-1 reservoir, recent work identified another one as the major source of replication-competent HIV-1: follicular helper $CD4^+$ T cells (T_{FH}) (Banga, R. [et al.], 2016).

1.2 Immune system

The innate immune system is the first line of host defense comprising multiple mechanisms and cells (macrophages, DCs and natural killer cells (NKs)) that provide immediate protection against infection. It is composed by physical and chemical barriers, such as skin and tears, and by complement system that helps in pathogens clearance (Turvey, S.E. and Broide, D.H., 2010). The main innate immune response is inflammation which is initiated by the recognition of pathogen structural motifs, known as pathogen-associated molecular patterns (PAMPs), by receptors present on some leukocytes (such as macrophages and DCs) – the pattern recognition receptors (PRRs), such as toll-like receptors (TLRs). This recognition allows cell activation and release of inflammatory cytokines, like TNF-α, IL-1 and interferon gamma (IFNγ) (Brubaker, S.W. [et al.], 2015).

Althought innate immune system provides a rapid response, it is not a long-lasting one and, more importantly, it is not pathogen-specific. Hence the evolutionary importance in acquiring a specific yet adaptable response able to prepare the host for future challenges and that generates an immunological memory enhancing the host response to subsequent encounter with the same pathogen – the adaptive immune system (Bonilla, F.A. and Oettgen, H.C., 2010).

The major players of adaptive immune system are B and T lymphocytes and their activation relies on antigen-presentation. This process is taken by all nucleated cells but there is a group of professional antigen-presenting cells (APCs) – DCs, macrophages and B cells – that provide a stronger immune response. While all nucleated cells are capable of presenting endogenous peptides (including the ones resulting from viral infections and/or expressed by cancer cells), APCs distinguish themselves by the ability of internalizing and processing exogenous antigens into peptides and displaying them complexed with MHC class II on the surface. While MHC class I is present in non-APCs and is recognized by T-cell receptor (TCR) expressed on the surface of cytotoxic T cells

($CD8^+$), MHC class II is present in APCs and is recognized via TCR expressed by helper T cells ($CD4^+$) (Bonilla, F.A. and Oettgen, H.C., 2010). Consequently, cytotoxic T cells are responsible for inducing programmed cell death to target cells (potentially harmful cells) (Berg, R.E. and Forman, J., 2006) and helper T cells have the crucial role of assisting other leukocytes, as it is the case of antibody secretion by B cells (Luckheeram, R.V. [et al.], 2012).

1.3 Follicular helper T cells (T_{FH})

Follicular helper T cells are a specialized subset of helper T cells that are mainly localized in germinal centers (GCs) within secondary lymphoid organs, as lymph nodes and tonsils. They play an important role in the formation and maintenance of GCs and generating lasting immune memory, which is fundamental for vaccination, considering that T_{FH} cells provide differentiation signals to B cells in order to secrete antibodies (Crotty, S., 2011).

1.3.1 T_{FH} differentiation

T_{FH} cell differentiation is a multifactorial process and it can be divided into three stages: APC-dependent, B cell interaction and GC T_{FH} cell (figure 1.5) (Crotty, S., 2014).

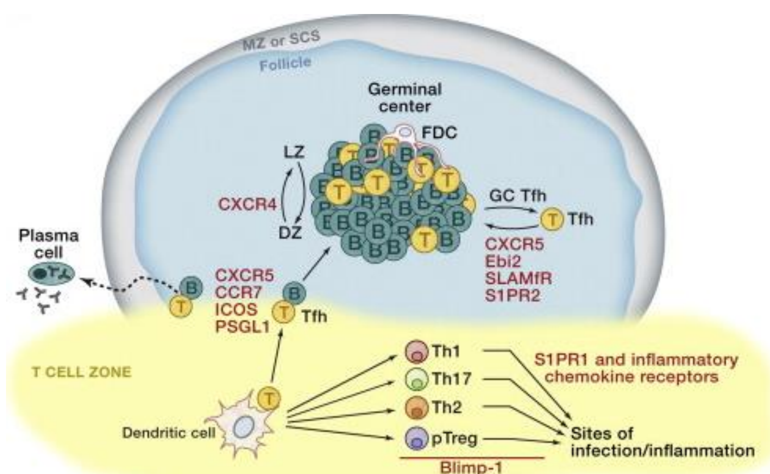

Figure 1.5 - T_{FH} cell differentiation. *From (Crotty, S., 2014)*

In the first stage, there is interaction between a naïve $CD4^{+}$ T cell and a dendritic cell in an antigen-presentation context, priming the $CD4^{+}$ T cell, in the T cell zone. Upon activation, it can differentiate into T_{FH} cell if it is $Bcl6^{+}$ (B cell lymphoma 6) which is an antagonist of another transcriptor factor Blimp-1, responsible for $CD4^{+}$ T cell differentiation into Th1, Th2, Th17 (T helper 1, 2 and 17 cells) (Crotty, S., 2011). Their differentiation also depends on IL-21, IL-6 and inducible co-stimulator (ICOS) (Jogdand, G.M. [et al.], 2016).

In the second stage, early-T_{FH} cell needs to migrate from T cell zone to T-B cell border. B cells express chemokines (C-X-C motif) ligand 13 (CXCL13) which are recognized by CXCR5 present on the early-T_{FH}. In addition to over-expressing CXCR5, early-T_{FH} cells downregulate CCR7 since it is the primary chemotactic receptor for the T-zone expressed chemokines (motif C-C) ligand 19 (CCL19) and 21 (CCL21) (Crotty, S., 2014).

The final stage is the germinal center formation where GC T_{FH} cells drive B cell differentiation into memory B cells and plasma cells and where antibody diversification (somatic hypermutation) occurs. This stage relies mainly on cytokine secretion by GC T_{FH}, such as IL-21 and IL-4, and surface protein CD40L which binds to CD40 expressed by GC B cells (Vinuesa, C.G. [et al.], 2016).

T_{FH} cells can also be found in peripheral blood althought they are less abundant under normal circumstances; in autoimmune disease or in infection context, they migrate from lymphoid tissues to peripheral blood. However, there is still some controversy whether lymphoid tissue T_{FH} cells play the same role than peripheral blood ones. Lymphoid tissue T_{FH} cells are commonly identified as high expressors of CXCR5 and programmed cell death protein 1 (PD-1) - $CXCR5^{high}PD\text{-}1^{high}$ (Yu, D. and Vinuesa, C.G., 2010).

1.3.2 T_{FH} cells as HIV reservoir

As stated before, T_{FH} cells have been described as the major reservoir for HIV active replication (Banga, R. [et al.], 2016, Lorenzo-Redondo, R. [et al.], 2016, Perreau, M. [et al.], 2013b) since they are primarily localized in an immune privileged site, considering that it is shielded from cytotoxic T cells, responsible for controlling HIV infection, and it is not accessible by ART as it does not permeate lymphoid tissue (Barton, K. [et al.],

2016, Paiardini, M. and Lichterfeld, M., 2016). Furthermore, these cells exhibit an enhanced susceptibility to HIV infection (about 40 times more than a non-GC cell) (Connick, E. [et al.], 2007) and they constitute 60-75% of HIV-producing cells (Connick, E. [et al.], 2007, Folkvord, J.M. [et al.], 2005). Moreover their lifespan is not affected by HIV infection. Actually, it has been reported that there is an expansion of this $CD4^{+}$ T cells subset (Lindqvist, M. [et al.], 2012). This GC permissitivity and expansion may be partially explained by the presence of highly infectious virions harbored by follicular dendritic cells (FDCs) that are chronically exposed to T_{FH} cells (figure 1.6) and by alterations in cytokine profile (as IL-6 and IFNγ) (Miles, B. and Connick, E., 2016).

However, there is still much to know about how HIV achieves this seemingly antagonic effects of increased viral replication and T_{FH} cell survival in order to maintain its own replication.

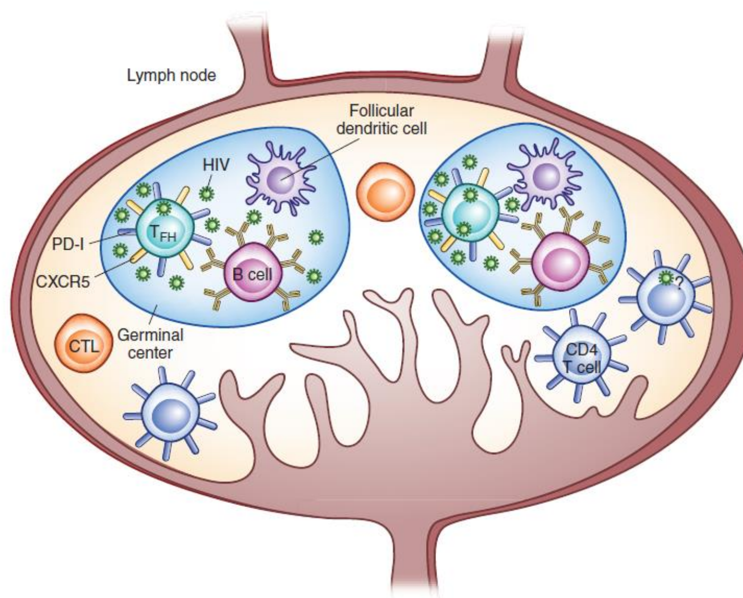

Figure 1.6 - HIV-1 persistence in GCs, during ART. *From (Paiardini, M. and Lichterfeld, M., 2016)*

1.4 Objectives

The work presented on this thesis was inserted in the project «HIV-1 replication on follicular sanctuaries», funded by Gilead (#PGG/009/2016) which aims at determining how HIV-1 manipulates T_{FH} microenvironment in order to improve its chances to thrive, without implicating T_{FH} cells survival, becoming a major reservoir. Ultimately, this project intends to provide the necessary tools to the pursuit of a functional cure for HIV-1 infection, by immunotherapy.

In the present work, the main goal is to get an insight of the nature of T_{FH} viral sanctuary by studying *ex vivo* HIV-1-infected T_{FH} cells isolated from healthy human tonsils. This constitute an advantage comparing with the previous studies that investigate T_{FH} compartment from HIV-1-infected patients as a whole, not allowing to discriminate between HIV-1 infection direct from bystander effects (Buranapraditkun, S. [et al.], 2017, Perreau, M. [et al.], 2013b).

By taking these first steps on understanding T_{FH} behavior, upon HIV-1 infection, it will arise many other questions that can lead to the identification of novel therapeutic targets.

2. Materials and Methods

2.1 Materials

2.1.1 General reagents and materials

Luria-Bertani (LB) Broth, Miller medium was obtained from Fisher BioReagents™, NucleoBond® Xtra Column Filters was from Macherey-Nagel, Dulbecco's modified Eagle's (DMEM) medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin (P/S) was from ThermoFisher as well as Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS and 1% P/S. Fluorescence-activated cell sorting (FACS) buffer is constituted by Phosphate-buffered saline 1X (PBS) from VWR and 2% of FBS from Biochrom. Fluorescence-activated cell sorting-Saponin (FACS-SAP) buffer is constituted by FACS buffer and saponin 0.1%. Biocoll (density 1,077 g/mL) used in density gradient cell separation was from Biochrom. For $CD4^+$ T cell isolation, MojoSort™ Human CD4 T cell isolation Kit from Biolegend was used. The MojoSort buffer is constituted by PBS 1X, 100 mM ethylenediamine tetraacetic acid (EDTA) and 5 mg/mL bovine serum albumin (BSA). For HIV-1 infection, it was used a ThermoMixer C for 15 mL tubes from Eppendorf.

2.1.2 FACS antibodies

Table 2.1 - Primary surface and intracellular antibodies used in FACS.

	Antibody	Isotype	Species	Supplier	Fluorochrome	Concentration (µg/ml)
Surface	CD4	IgG1	Mouse	Biolegend	PerCP-Cy5.5	100
	CXCR5	IgG1	Mouse	Biolegend	APC-Cy7	200
		IgG1	Mouse	Biolegend	PE	100
	PD-1	IgG1	Mouse	Biolegend	BV421	100
		IgG1	Mouse	Biolegend	PE-Cy7	200
	CD3	IgG2a	Mouse	Biolegend	APC-Cy7	200
		IgG1	Mouse	Biolegend	PerCP	100
	ICOS	IgG	Hamster	Biolegend	APC	200
	CD38	IgG1	Mouse	Biolegend	APC-Cy7	400
	CD69	IgG	Hamster	Biolegend	PE	200
Intracellular	Ki67	IgG1	Mouse	BD Pharmog	PE-Cy7	100
	Bcl6	IgG2	Rat	Biolegend	PE-Cy7	25
	IL-17	IgG1	Mouse	Biolegend	APC-Cy7	100

	IL-21	IgG1	Mouse	Biolegend	A647	80
	IFNγ	IgG1	Mouse	Biolegend	Pacific-Blue	100
	IL-10	IgG1	Rat	Biolegend	A647	100
	TLR7	IgG1	Mouse	Novus	PE	700

2.1.3 FACS dyes

Table 2.2 - Dyes used in FACS

Dye	Supplier	Fluorochrome
LIVE/DEAD™ Fixable Aqua	Invitrogen	BV510
MitoTracker® Deep Red FM	Invitrogen	A647

2.2 Methods

2.2.1 Viral plasmid production

2.2.1.1 Bacterial transformation

1 µL of viral DNA (HIV-NL-4-3 (Silva, J.G. [et al.], 2016) or NLENG1-IRES (Trinite, B. [et al.], 2013), both containing a green fluorescent protein (GFP) reporter gene) was added to DH5α competent cells and incubated on ice for 20 min. The mix was placed in a water bath at 42ºC, for 45 sec and placed back in ice for 2 min. 1 mL LB medium was added to the mix, incubated in a shaking incubator at 37ºC, 0,45 x g for 1 h and centrifuged at 3200 x g for 5 min. The mix was incubated overnight (O/N), at 37ºC, in a 10 cm LB-agar plate supplemented with 100 µg/mL ampicillin.

2.2.1.2 Bacterial inoculation

A single bacterial colony was pre-inoculated at 37ºC, 0.45 x g for 8 h (logarithmic phase of bacterial growth) in 5 mL LB medium supplemented with 100 µg/mL ampicillin. Pre-inoculum was placed in 200 mL LB medium supplemented with 100 µg/mL ampicillin and incubated O/N at 37ºC, 0.45 x g (saturation phase of bacterial growth).

2.2.1.3 Viral DNA purification

Bacterial culture was centrifuged at 800 x g for 30 min. DNA was recovered from bacterial culture's pellet with NucleoBond® Xtra Column Filters by anion-exchange chromatography, according to manufacturer's instructions (Macherey-Nagel). Viral DNA was resuspended in 100 µL milliQ water and stored at -20ºC.

2.2.2 Viral particles production

2.2.2.1 Transfection

293T packaging cells were seeded O/N at 37ºC, 5% CO_2, at $4x10^6$ cells in a 10 cm plate in 10 mL supplemented DMEM medium. They need to grow until they reach 70-90% confluency before transfection. A mix with 20 µg of packaging plasmid (HIV-NL4-3) and 20 µg of envelope plasmid (ENV-VSV) or 40 µg of whole plasmid (NLENG1-IRES), $CaCl_2$ and milliQ water (to a final volume of 1 mL) was added dropwise to 1 mL Hepes-buffered saline (HBS) 2X, while bubbling it. The final mix was added dropwise to 293T cells (1 mL/10 cm plate). The packaging cells were incubated O/N at 37ºC, 5% CO_2. Media was changed at 24 h.

2.2.2.2 Supernatant collection

Viral particles (supernatant) were collected with a 10 mL syringe, filtered through a 0.45 µm filter and stored at -80 ºC, at 48 h post-transfection.

2.2.3 $CD4^+$ T cells isolation from human tonsils

2.2.3.1 Human tonsils processing

The human tonsils were gently provided by Professor Luís Graça, from Instituto de Medicina Molecular (iMM), in the scope of a protocol established between this laboratory and Hospital de Santa Maria, in Lisbon. The human tonsils were placed in FACS buffer (PBS 1X with 2% FBS) in a 10 cm plate and cauterized parts and inflamed tissue were

removed, the remaining tissue was mechanically disintegrated and was filtered with a 100 µm mesh. Then, it was diluted (1:2) with PBS 1X, added carefully on top of Biocoll (1:3) and centrifuged at 700 x g for 30 min (without brake) for density gradient cell separation. The mononuclear cells (MNCs) ring was carefully collected and washed two times with PBS 1X at 700 x g for 10 min.

2.2.3.2 $CD4^+$ T cell isolation

For $CD4^+$ T cell isolation, it was followed the MojoSort™ Isolation Kits No Wash Protocol. They were culture with supplemented RPMI medium at $2x10^6$ $CD4^+$ T cells/mL, O/N at 37ºC, 5% CO_2.

2.2.4 $CD4^+$ T cells HIV-1 infection

On the next day, $CD4^+$ T cells were centrifuged, the supernatant was removed and they were resuspended with HIV-1 (500 µL HIV-NL4-3/million cells or 75 µL NLENG1-IRES/million cells) and 15 µg/mL of polybrene, in 15 mL tubes. They were placed in a Thermo Mixer C, for 4 h, at 37ºC with agitation cycles of 300 rpm for 1 min every 5 mins. Then, they were incubated for another 4 h at 37ºC, 5% CO_2. After that time, cells were centrifuged at 300 x g for 5 min, resuspended in supplemented RPMI medium at $2x10^6$ $CD4^+$ T cells/mL and cultured for 3 days at 37ºC, 5% CO_2.

2.2.5 TLR7 stimulation

For TLR7 stimulation in non-infected $CD4^+$ T cells, it was used its synthetic agonist Imiquimod (IMQ) (1 mg/mL, from InVivoGen) at 0, 0.5, 1, 2.5, 5 and 10 µg/mL. $CD4^+$ T cells were culture at $2.5x10^6$/mL, for 3 days at 37ºC, 5% CO_2.

2.2.6 Data acquisition and processing

2.2.6.1 Flow cytometry

Uninfected and infected $CD4^+$ T cells were washed with PBS 1X (at 700 x g for 3 min) and they were stained with MitoTracker® Deep Red FM and posteriorly with LIVE/DEAD™ Fixable Aqua, following manufacturer's instructions. For surface staining, cells were washed twice with FACS buffer and incubated for 20 min, at RT (room temperature), in the dark, with the surface antibodies (table 1). This staining was followed with two washes with FACS buffer in order to remove the unbound antibodies. Cells were fixed with paraformaldehyde (PFA) 1%, for 20 min, at RT, in the dark and then washed with FACS buffer. For membrane permeabilization, it was used FACS-SAP with the same incubation's condition as cell fixation. For intracellular staining, cells were centrifuged at 700 x g for 3 min and incubated for 30 min, at RT, in the dark, with the intracellular antibodies (table 1). Finally, cells were washed twice with FACS-SAP and once with FACS buffer and the data was acquired in FACS Canto II from BD Biosciences. The data was analyzed with Flow Jo software (BD Biosciences).

2.2.6.2 Statistical analysis

GraphPad Prism software was used for statistical analysis. Statistical significance (p-values) was obtained using Wilcoxon matched-pairs test; p values of less than 0.05 were considered significant.

3. Results

3.1 GC T_{FH} population identification

After CD4+ T cells isolation from human tonsils, GC T_{FH} population was identified by flow cytometry, following the gating strategy represented on figure 3.1. In addition to this population, that have been described as PD-1^{high}CXCR5^{high} (Yu, D. and Vinuesa, C.G., 2010), it is also possible to identify non-GC T_{FH} (nGC T_{FH}), which are intermediate expressors, and non-T_{FH} cells (nT_{FH}) which are considered as PD-1^{-}CXCR5^{-}.

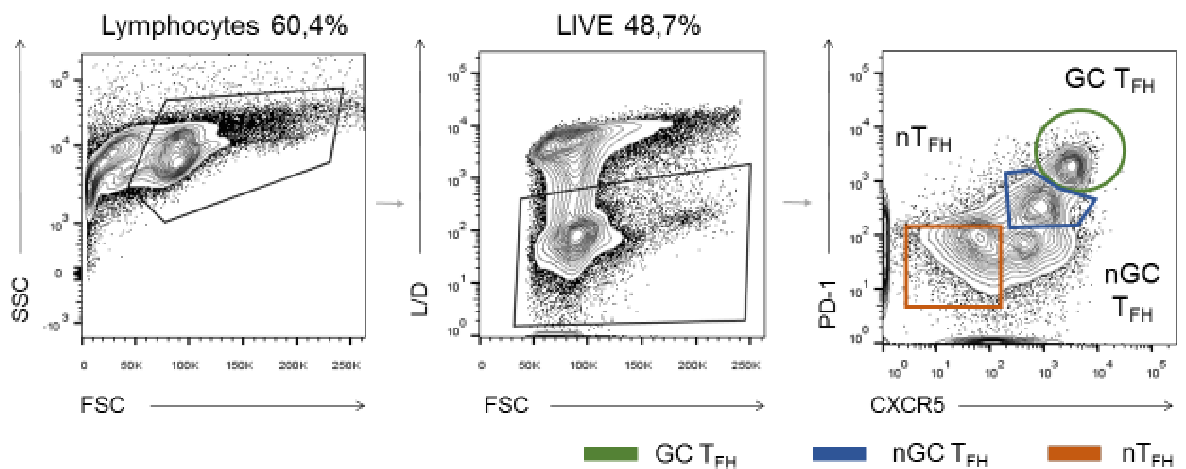

Figure 3.1 – Gating strategy used for GC T_{FH} identification (CD4+PD-1^{high}CXCR5^{high} T cells), by flow cytometry. GC T_{FH} (green) represents 10.7%, nGC T_{FH} (blue) represents 15.4% and nT_{FH} (orange) represents 22% of tonsils CD4+ T cells.

3.2 Tonsils CD4+ T cells populations susceptibility to HIV-1

After having identified these three different tonsils cells populations (GC T_{FH}, nGC T_{FH} and nT_{FH}), the next question was: are they equally susceptible to HIV-1 infection? The answer is no: GC T_{FH} population appears to be more susceptible to HIV-1 infection (10.9%) than nGC T_{FH} (8.16%) and nT_{FH} population (3.25%) (figure 3.2 B). Curiously, HIV-1 infection increases the viability of infected cells (52%) (figure 3.2). CD4 receptor downregulation is one of the HIV infection hallmarks and it is visible in all infected populations in figure 3.2 B.

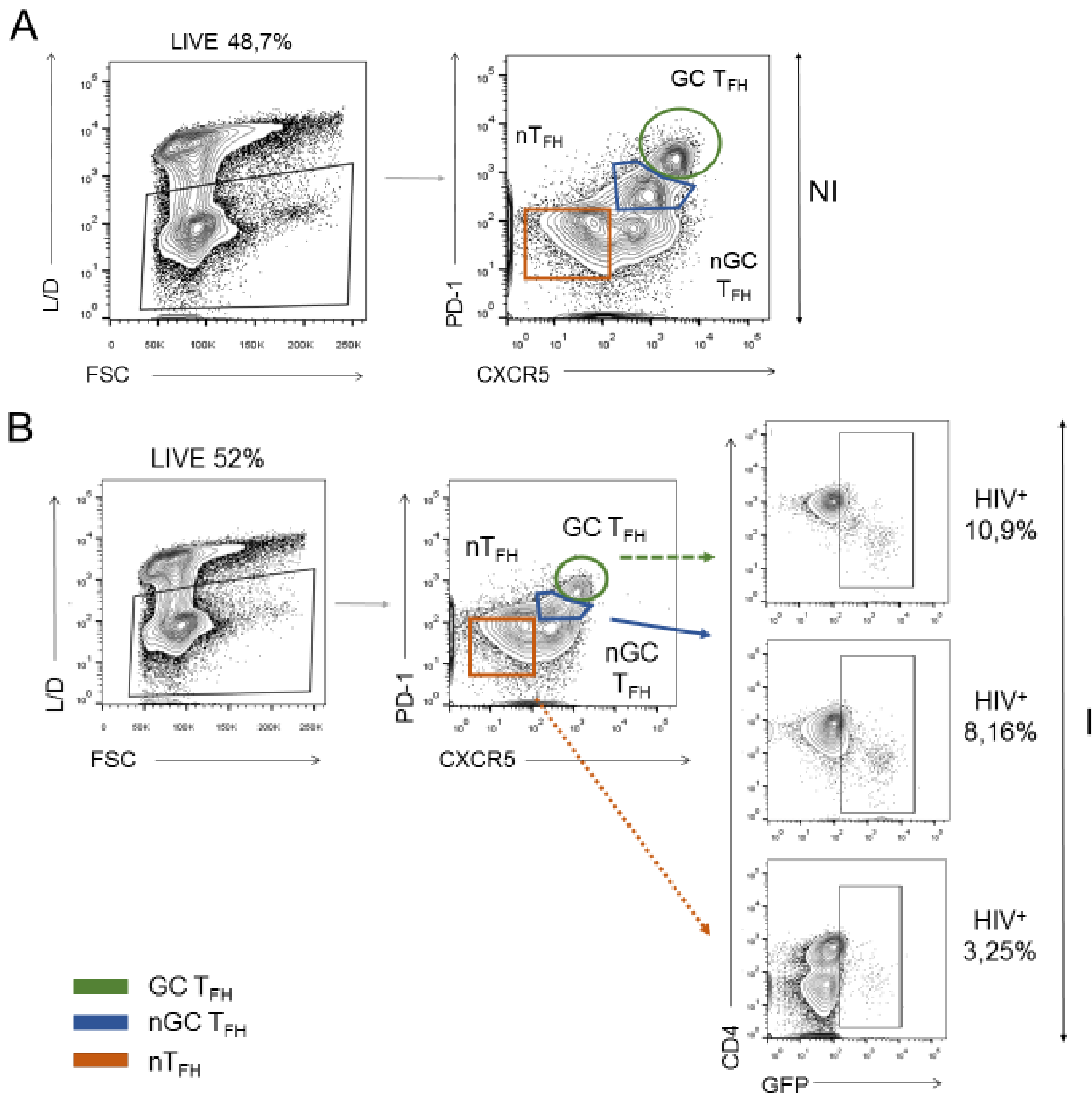

Figure 3.2 – Tonsils $CD4^+$ T cells populations susceptibility to HIV-1, by flow cytometry, gated on "Lymphocytes" following the gating strategy represented on figure 3.1. **(A)** Non-infected cells (NI). GC T_{FH} represents 10.7%, nGC T_{FH} represents 15.4% and nT_{FH} represents 22% of tonsils $CD4^+$ T cells. **(B)** Infected cells (I). GC T_{FH} represents 6.16%, nGC T_{FH} represents 7.83% and nT_{FH} represents 22% of tonsils $CD4^+$ T cells.

3.3 HIV-1 effects on GC T_{FH} cells

3.3.1 HIV-1 expands T_{FH} population

PD-1, CXCR5 and transcription factor Bcl6 are known T_{FH} markers. Analyzing both mean fluorescence intensity (MFI) of Bcl6 and the percentage of Bcl6-expressors T_{FH} cells (figure 3.3 A), despite the presence of an outlier donor, it is visible a slight increase on this transcription factor upon HIV-1 infection. This virus also seems to increase CXCR5 (figure 3.4) leading to the proposal that HIV-1 drives T_{FH} differentiation (Bcl6 increase) and recruitment (CXCR5 increase). Although there are some evidences that HIV-1 decreases the PD-1 inhibitory signaling (Kohler, S.L. [et al.], 2016); in this study, only half of the donors exhibited that feature, rendering these results inconclusive.

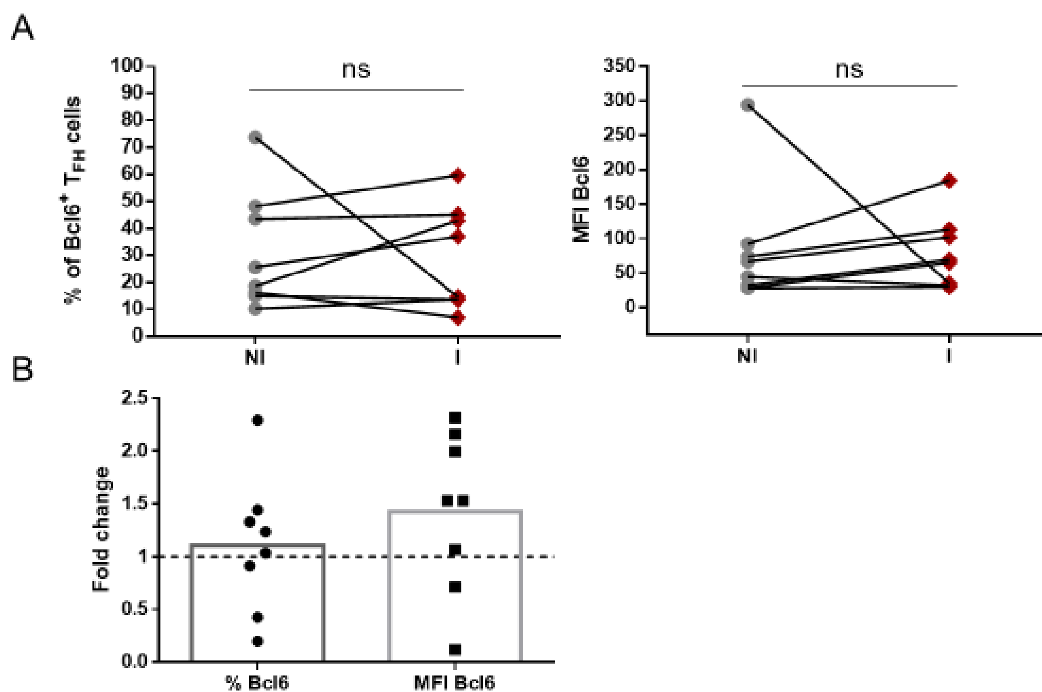

Figure 3.3 – HIV-1 increases the expression of the lineage specific transcription factor Bcl6 in GC T_{FH} cells. **(A)** Percentage (left) and MFI (right) of $Bcl6^+$ NI and HIV-1-infected GC T_{FH} (I). **(B)** Fold change of the percentage and MFI of $Bcl6^+$ GC T_{FH} cells upon HIV-1 infection. Grey bars represent the median. Each symbol represents an individual donor. p values were determined by Wilcoxon matched-pairs test; ns, not significant ($p > 0.05$).

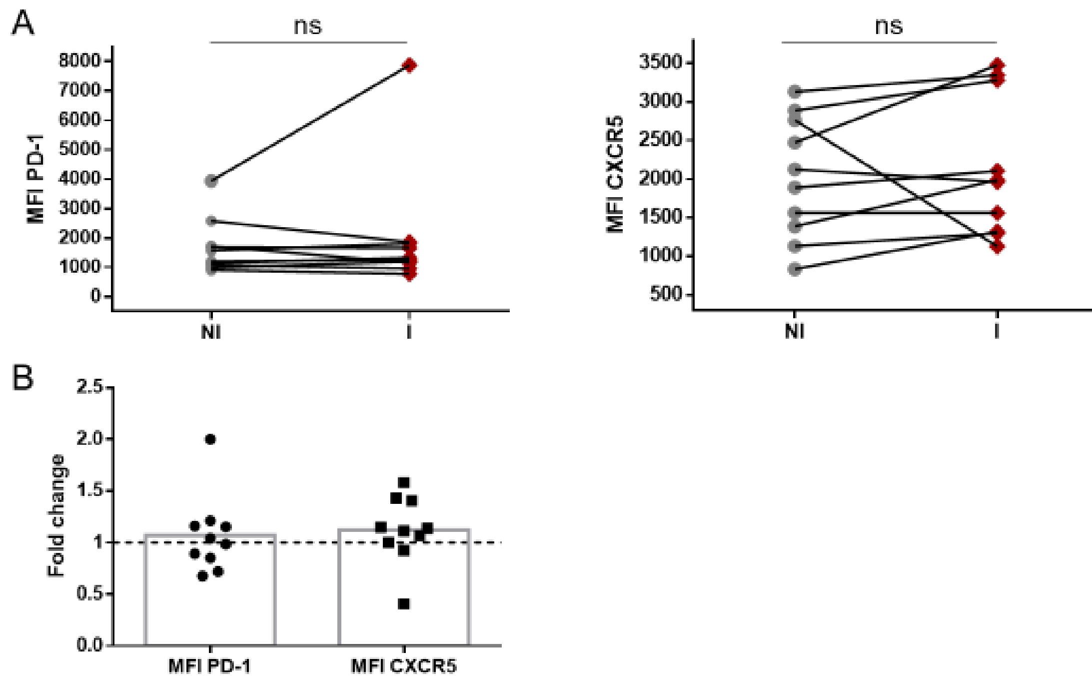

Figure 3.4 – HIV-1 seems to increase GC T_{FH} chemotactic receptor CXCR5. **(A)** MFIs of PD-1 (left) and CXCR5 (right) from NI and HIV-1-infected GC T_{FH} cells. **(B)** Fold change of MFIs of PD-1 and CXCR5 upon HIV-1 infection. Grey bars represent the median. Each symbol represents an individual donor. p values were determined by Wilcoxon matched-pairs test; ns, not significant ($p > 0.05$).

3.3.2 HIV potentially augments GC T_{FH} cell survival

Since other papers have described a role to bystander HIV-1 effects in expanding GC T_{FH} cells (Buranapraditkun, S. [et al.], 2017, Perreau, M. [et al.], 2013b), it would be interesting to determine if HIV-1 could direct the proliferation of the infected GC T_{FH} cells. Unexpectedly, HIV-1 infection directly increases GC T_{FH} cell proliferation (measured by nuclear protein Ki67), as seen in the percentage of $Ki67^+$ T_{FH} cells (figure 3.5 A) and in an approximately 6-fold change (figure 3.5 B).

Both viral replication and higher proliferation observed in HIV-1 infected GC T_{FH} impose high demands for energy and biosynthetic precursors. For that reason, the following step was to check whether HIV-1 could affect GC T_{FH} cell metabolism by measuring the mitochondrial mass (Mitotracker). HIV-1 infected GC T_{FH} cells increase their mitochondrial mass to meet the higher energy demands required for HIV replication and for their increased cellular proliferation (figure 3.6). Finally, it was observed an increase in the activation status of HIV-1-infected GC T_{FH} cells (measured by transmembrane protein CD69) (figure 3.7).

These results raise the intriguing possibility that HIV-1 may promote its replication in GC T_{FH} cells in two ways: one, by increasing the viral production output, and, more unconventionally, by driving the proliferation of the infected cells, turning them into expandable bioreactors.

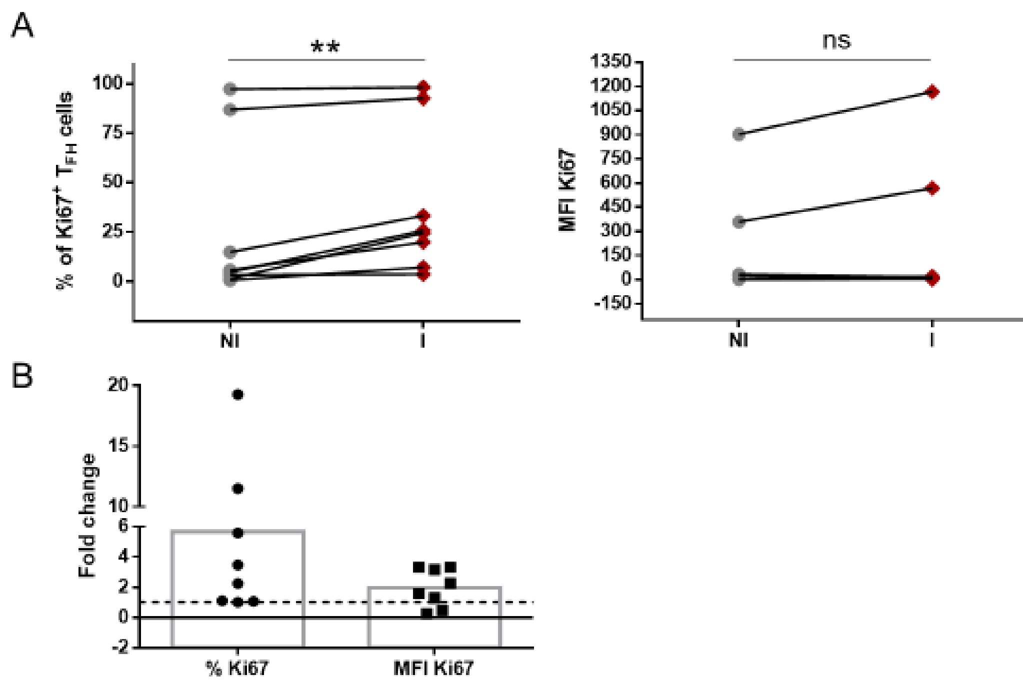

Figure 3.5 – HIV-1 increases GC T_{FH} cell proliferation. **(A)** Percentage (left) and MFI (right) of $Ki67^+$ NI and HIV-1-infected GC T_{FH} cells. **(B)** Fold change of percentage and MFI of $Ki67^+$ GC T_{FH} cells upon HIV-1 infection. Grey bars represent the median. Each symbol represents an individual donor. p values were determined by Wilcoxon matched-pairs test; ns, not significant ($p > 0.05$), ** $p < 0.01$.

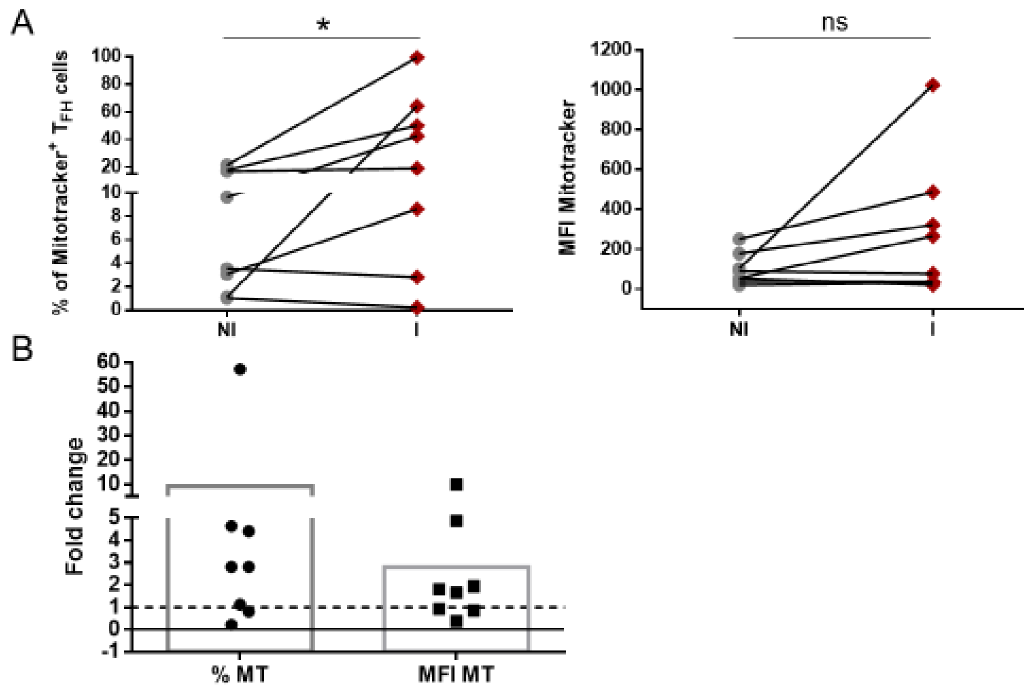

Figure 3.6 – HIV-1 imposes an increased GC T_{FH} metabolism. **(A)** Percentage (left) and MFI (right) of MT^+ (Mitotracker$^+$) NI and HIV-1-infected GC T_{FH} cells. **(B)** Fold change in the percentage and MFI of MT^+ GC T_{FH} cells upon HIV-1 infection. Grey bars represent the median. Each symbol represents an individual donor. p values were determined by Wilcoxon matched-pairs test; ns, not significant ($p > 0.05$), * $p < 0.05$.

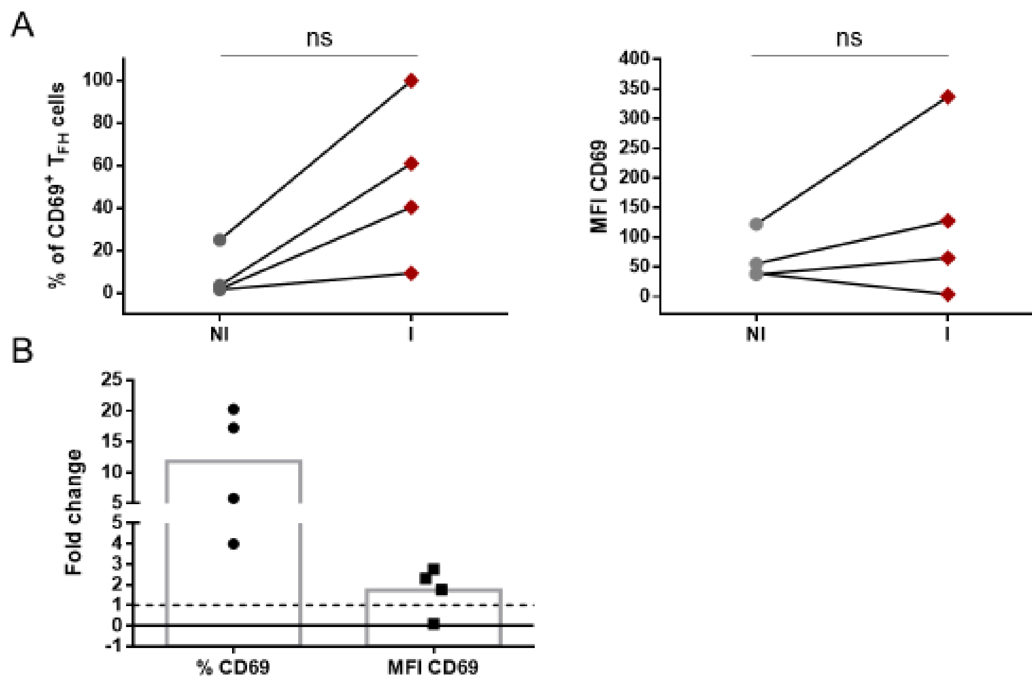

Figure 3.7 – HIV-1 potentiates an activation status of GC T_{FH} cells. **(A)** Percentage (left) and MFI (right) of $CD69^+$ NI and HIV-1-infected GC T_{FH} cells. **(B)** Fold change of percentage and MFI of $CD69^+$ GC T_{FH} cells upon HIV-1 infection. Grey bars represent the median. Each symbol represents an individual donor. p values were determined by Wilcoxon matched-pairs test; ns, not significant ($p > 0.05$).

3.3.3 HIV-1 modulates GC T_{FH} cell signaling

Bearing in mind that HIV-1 seemingly enhances T_{FH} metabolism and, consequently, proliferation, the virus is also likely to control T_{FH} signaling. Accordingly, HIV-1 slightly upregulates TCR (CD3) in GC T_{FH} cells (figures 3.8 A and B), possibly to increase T_{FH} activation.

Regarding ICOS expression, there is a decrease in the percentage of expressing-T_{FH} cells (figure 3.8 A) which is corroborated by the fold decrease (figure 3.8 D) although there is a subtle upraise of their ICOS expression (figure 3.8 B and D). However, ICOS expression was highly related with donor-specific characteristics, producing inconsistent outcomes.

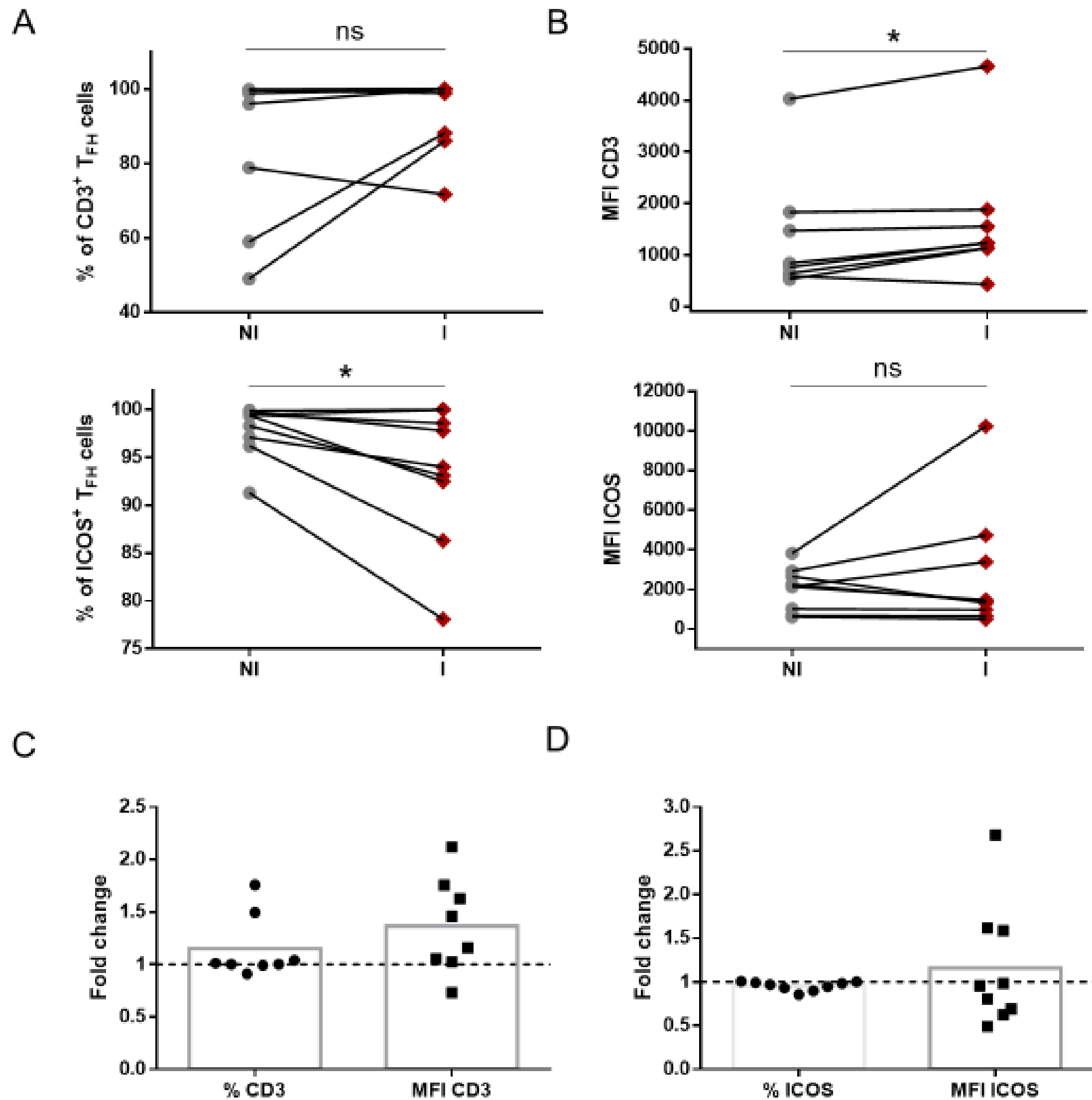

Figure 3.8 – HIV-1 apparently modulates GC T_{FH} signaling. **(A)** Percentage of $CD3^+$ (top) and $ICOS^+$ (bottom) NI and HIV-1-infected GC T_{FH} cells. **(B)** MFIs of CD3 (top) and ICOS (bottom) in NI and HIV-1-infected GC T_{FH} cells. Fold change of percentage and MFI of **(C)** $CD3^+$ and **(D)** $ICOS^+$ GC T_{FH} cells upon HIV-1 infection. Grey bars represent the median. Each symbol represents an individual donor. p values were determined by Wilcoxon matched-pairs test; ns, not significant ($p > 0.05$), * $p < 0.05$.

3.3.4 TLR7 modulated signaling is HIV-1 specific

TLRs act as sensors of PAMPs expressed on infectious agents, providing a positive co-stimulatory signal that induces pro-inflammatory cytokines and cell proliferation. There are surface and endosomal TLRs, allowing a broad pathogen recognition and immune response. Among several receptors, there is an intracellular one capable of recognize ssRNA, such as HIV-1 – the TLR7.

So far, this work demonstrated that HIV-1 modulates GC T_{FH} cells signaling aiming at improving this cellular reservoir capacities. Therefore, it is imperative to understand the extent of HIV-1 influence on T_{FH} cellular machinery. In fact, HIV-1-infected GC T_{FH} cells present a higher expression of TLR7 (figure 3.9).

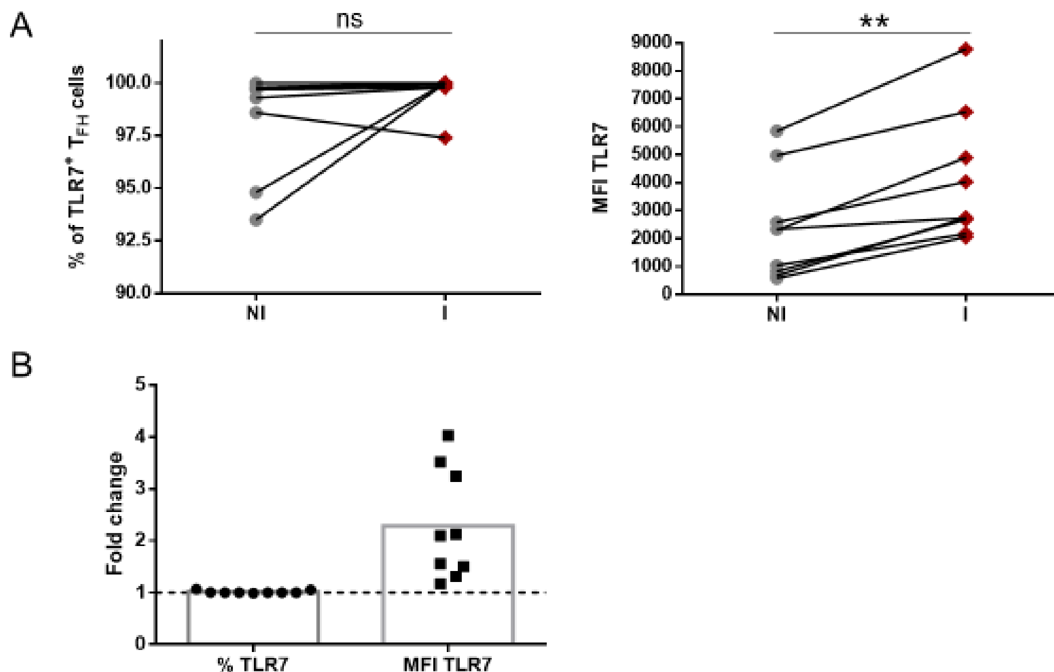

Figure 3.9 – HIV-1 increases TLR7 expression in GC T_{FH} cells. **(A)** Percentage (left) and MFI (right) of $TLR7^+$ NI and HIV-1-infected GC T_{FH} cells **(B)** Fold change of percentage and MFI of $TLR7^+$ GC T_{FH} cells upon HIV-1 infection. Grey bars represent the median.

Each symbol represents an individual donor. p values were determined by Wilcoxon matched-pairs test; ns, not significant ($p > 0.05$), ** $p < 0.01$.

In order to determine if the upregulation of TLR7 was a simple response to ligand abundance, a TLR7-synthetic agonist Imiquimod (IMQ) was added to non-infected cells. Strikingly, IMQ induced different outcomes comparing with HIV-1-infected cells. Upon IMQ stimulation, there is a trend for TLR7 expression decrease in NI T_{FH} cells (figure 3.10), more pronounced for the highest IMQ concentration (10 μg/mL), indicating cellular defensive mechanism (negative feedback) to avoid chronic stimulation. Additionally, IMQ decreases GC T_{FH} cells proliferation (figure 3.11).

Altogether, these data show that HIV-1 may induce an alternative signaling pathway of TLR7 in order to escape a cellular protective response and to allow its cellular reservoir expansion.

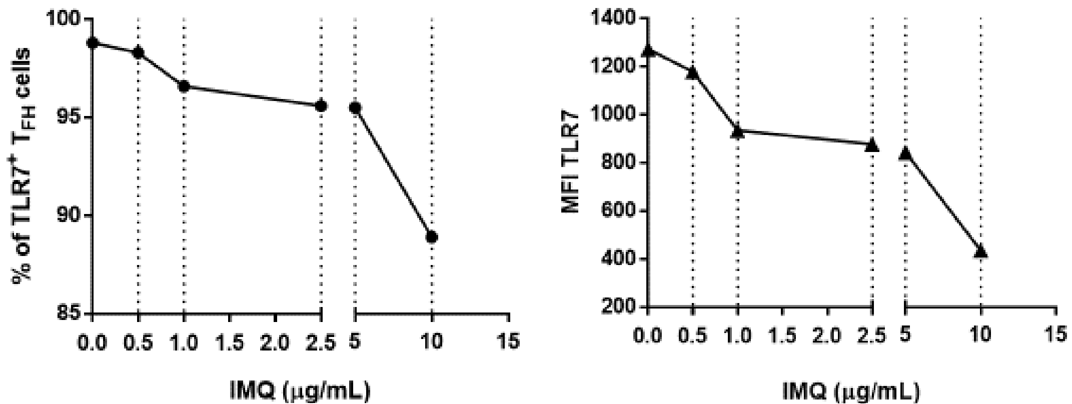

Figure 3.10 – IMQ decreases TLR7 expression in NI GC T_{FH} cells. Percentage (left) of $TLR7^{+}$ T_{FH} cells and MFI (right) of TLR7 from NI GC T_{FH}, for each IMQ concentration used (0, 0.5, 1, 2.5, 5 and 10 μg/mL).

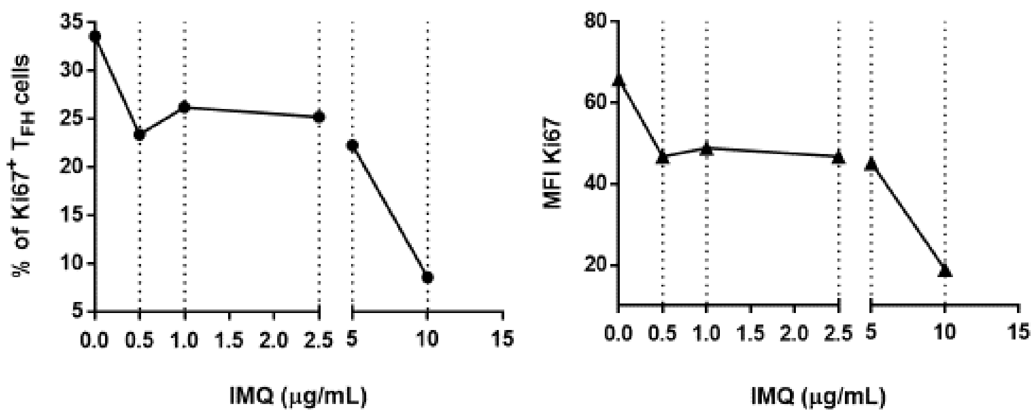

Figure 3.11 – IMQ decreases proliferation capacity of NI GC T_{FH} cells. Percentage (left) and MFI (right) of $Ki67^{+}$ NI GC T_{FH} cells, for each IMQ concentration used (0, 0.5, 1, 2.5, 5 and 10 μg/mL).

3.3.5 GC T_{FH} cytokine profile may be shaped by HIV-1

A recent work demonstrated that TLR7 engagement by HIV-1, in circulating $CD4^{+}$ T cells, induces a state of anergy and an inability to secrete cytokines (Dominguez-Villar, M. [et al.], 2015). In the light of the previous results, it was important to check if this hold true to the present work. For that reason, the pro-inflammatory cytokines IL-21, IL-17 and IFNγ were measured in GC T_{FH}, without any stimulation, and compared with HIV-1-infected ones, knowing that they present an amplified TLR7 expression.

The T_{FH} cytokine profile seems to be affected by HIV-1 infection, since it is visible an expansion of $IL\text{-}21^{+}$, $IL\text{-}17^{+}$ and $IFN\gamma^{+}$ T_{FH} cells (figure 3.10 A and B) and in their expression levels (figure 3.10 C), even though it is more evident in IL-21 and IFNγ considering their major role in directing antibody production. Hence, contrary to circulating $CD4^{+}$ T cells, HIV-1 does not induce anergy in GC T_{FH} cells.

All points out to an infection mechanism, orchestrated by HIV-1, aiming at improving its replication and reservoir maintenance in GC T_{FH} cells, through metabolism and signaling modulation.

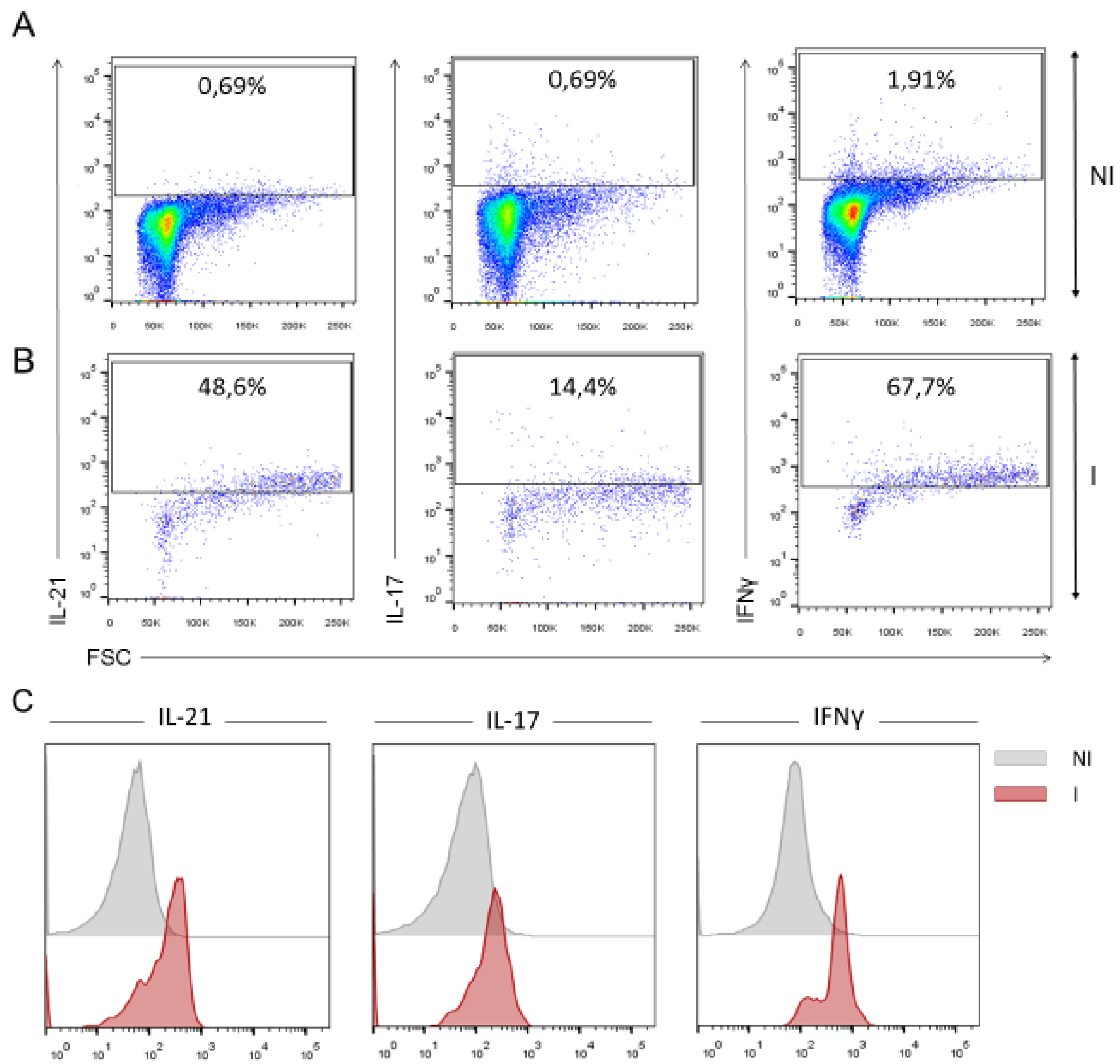

Figure 3.12 – HIV-1 interferes with GC T_{FH} cytokine production. Percentage of IL-21$^+$ (left), IL-17$^+$ (middle) and IFNγ$^+$ (right) **(A)** NI and **(B)** HIV-1-infected GC T_{FH} cells. **(C)** MFIs of described cytokines in NI (grey) and HIV-1-infected (red) GC T_{FH} cells.

4. Discussion

The combination antiretroviral therapy, introduced in 1996, has been one of the most outstanding achievements of medical research for the treatment of HIV infection, preventing millions of AIDS-related deaths and improving HIV-infected individual's life expectancy. However, it is unable to clear persistent HIV reservoirs which remain the major obstacle to a functional cure and enforces a life time of treatment (Chun, T.W. [et al.], 2015). Such therapy entails a number of serious side effects such as heart and liver diseases and drug interactions (Marzolini, C. [et al.], 2011). Moreover, a life time of ART leads to development of drug resistance, an evasion mechanism in which occurs mutations in ART-targeted viral proteins, resulting in a great genetic variation within HIV populations. This phenomenon poses a real concern since it generates a high-fitness virions population capable of evade treatment (Clavel, F. and Hance, A.J., 2004). In fact, it is estimated that, in the United States of America, 76% of ART-treated patients developed resistance to one or more antiretroviral drugs (Richman, D.D. [et al.], 2004). That is why a therapy targeting the host signaling instead of the virus offers, not only the advantage of minimize the described selective pressure on HIV, but also the opportunity to compile a library of signaling inhibitors that, unlike ART, are capable of permeate lymphoid tissue (the major HIV-persistent reservoir).

Follicular helper T cells constitute the most important cellular reservoir for HIV-1 persistence and replication (Paiardini, M. and Lichterfeld, M., 2016). As described previously in HIV-1-infected patients (Perreau, M. [et al.], 2013b), they exhibited an enhanced susceptibility to HIV-1 infection compared to non-follicular helper T cells or even non-germinal center ones (figure 3.2 B). In addition to that, GC T_{FH} cells display a longer lifespan upon infection (figure 3.2) and an increased differentiation and recruitment, by the upregulation of T_{FH} transcription factor Bcl6 and the chemokine receptor CXCR5, respectively (figures 3.3 and 3.4). These data suggest that HIV-1 preferentially drives GC T_{FH} population expansion.

Furthermore, HIV-1 augments GC T_{FH} cells proliferation and potentiate an activated state (figures 3.5 and 3.7) in order to favor viral replication. These events impose a higher demand for energy that is visible by the increased mitochondrial mass in infected cells (figure 3.6). The essential role of T_{FH} cells in GCs requires a mixed metabolism. It is

known that T_{FH} cells rely both on glycolysis and mitochondrial oxidative phosphorylation (Ray, J.P. [et al.], 2015, Zeng, H. [et al.], 2016), being the last one the most efficient pathway in energy generation but glycolysis enables "building blocks" formation which is crucial for viral particles production. In a previous study, it was demonstrated that HIV-1-infected circulating $CD4^+$ T cells display an upregulated glucose transporter Glut1 expression meaning that HIV-1 most likely shifts the metabolism towards glycolysis (Loisel-Meyer, S. [et al.], 2012). Nevertheless, whether HIV-1 reprograms GC T_{FH} metabolism towards one or the other metabolic pathway and, if so, which metabolic factors are involved, is still unknown. Combining the data obtained in the present work with the data from Loisel-Meyer, S. [et al.], 2012, it rises the idea that HIV-1 shapes T_{FH} metabolism by activating the oxidative phosphorylation metabolic pathway to fulfill the energetic needs along with upregulation of the glycolytic pathway to provide "building blocks" to sustain a highly active HIV-1 replication.

Even though HIV-1 replication also rely on an activated cellular state (Stevenson, M. [et al.], 1990), there is still much to know about how this virus affects TCR signaling in T_{FH} cells. It is described that the increased pathogenicity observed in HIV-1 results in a loss of capacity of Nef protein to downregulate TCR-CD3, as seen in less pathogenic HIV strains (Feldmann, J. [et al.], 2009, Schindler, M. [et al.], 2006) where it act as a host protective measure by ensuring a minimal cellular activation and, consequently, a reduced activation-induced T cell death (Foster, J.L. and Garcia, J.V., 2006). Knowing that GC T_{FH} cells are in an activated and proliferative state, upon HIV-1 infection, it is not surprising that TCR-CD3 is upregulated (figure 3.8 A).

ICOS co-stimulatory signal is crucial for T_{FH} differentiation (Rolf, J. [et al.], 2010) and it may play a role in HIV-1 infection since it highly activates PI3K/Akt/mTOR signaling pathway, responsible for cell activation and proliferation (Wikenheiser, D.J. and Stumhofer, J.S., 2016). However, in this study, it was not possible to determine if this idea is correct since ICOS expression was donor-related. On the other hand, PD-1 suppresses cell signaling promoting apoptosis and it was previously described that HIV-1 downmodulates this inhibitor in order to ensure its own replication on the host cells (Kohler, S.L. [et al.], 2016). Nevertheless, it was not possible to conclude the effect of HIV-1 infection in GC T_{FH} cells, in the present study, since only half of the donors exhibited that downmodulation, exposing the need to test in more donors.

Toll-like receptors represent a critical role in activating an innate immune response, TLR7, in particular, is responsible for ssRNA recognition in intracellular compartments. This study showed that HIV-1 enhances TLR7 expression in GC T_{FH} cells (figure 3.9) indicating a modulated TLR signaling in order to promote HIV-1 expansion. These data raise questions regarding the altered T_{FH} signaling by HIV-1 that need to be answered in the near future.

Although TLR engagement act as a positive co-stimulatory signal to increase pro-inflammatory cytokines secretion and cell proliferation, it was demonstrated that, in circulating HIV-1-infected $CD4^+$ T cells, it induces an anergic state (Dominguez-Villar, M. [et al.], 2015). This does not appear to apply to GC T_{FH} cells considering the increased pro-inflammatory cytokines expression in HIV-1-infected cells (figure 3.12). This data also corroborates the chronic state of inflammation as a HIV-1 infection hallmark.

Altogether, these data indicate a metabolic control and signaling modulation, by HIV-1 as a disease mechanism, in order to turn GC T_{FH} cells into viral factories. This work demonstrates the urgent need to gain more insight about how HIV-1 exploits T_{FH} cell machinery (crosstalk of TCR, TLR7 and cytokines) aiming at discovering crucial therapeutic targets to achieve a functional cure for HIV-1 infection.

5. Conclusions and future perspectives

The present work provided the framework for further investigation in T_{FH} cellular machinery upon HIV-1 infection, by demonstrating an enhanced T_{FH} differentiation, proliferation and survival at the expense of an altered metabolism (increased oxidative phosphorylation metabolic pathway) and signaling (increased TCR, TLR7 and pro-inflammatory cytokines).

Nonetheless, not only there is still serious gaps of knowledge regarding GC T_{FH} cells signaling and metabolism, but also concerning the HIV-1 effects on them. It is imperative to know which signaling events/molecules are altered due to infection as well as the effect of PD-1 inhibitory and ICOS co-stimulatory signals both on TCR activation and on T_{FH} cell function, since it was described an inadequate B cell help upon PD-1 engagement in HIV-1-infected cells (Cubas, R.A. [et al.], 2013). In addition, there is the need to investigate the possible crosstalk between TCR and TLR7, along with cytokine profile, and their consequences on viral and antibody production.

Since it was proved that HIV-1 is able to manipulate T_{FH} metabolism, it is important to check whether it favors a single metabolic pathway and which metabolic molecules are involved in order to selectively inhibit metabolic pathways hijacked by HIV-1 instead of targeting general ones, preventing the harmful of functional cells.

Ultimately, by understanding the HIV-1 infection mechanism in this cellular reservoir, will lead to the identification of novel and more accurate therapeutic targets which brings us closer to a cure to HIV-1 infection.

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