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HIV-2 ability to infect Human Follicular T helper cells

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ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
APC	Antigen-presenting cells
ART	Antiretroviral therapy
BATF	Basic leucine zipper transcriptional factor ATF-like
Bcl-6	B cell lymphoma 6
BCR	B-cell receptor
Blimp-1	B lymphocyte-induced maturation protein-1
BSA	Bovine serum albumin
BSL3	Biosafety Laboratory level 3
CA	(viral) Capsid
CCR	C-C chemokine receptor
cDNA	Complementary DNA
CD40	Cluster of differentiation 40
CD40L	CD40 ligand
CM	Complete medium
CPRG	Chlorophenol red-β-D-galactopyranoside
CXCR	C-X-C chemokine receptor
DC	Dendritic cells
DMEM	Dulbecco's Modified Eagle Medium
DNA	Desoxiribonucleic acid
dNTP	Deoxyribonucleotide trisphosphate
ECs	Elite controlers
FDC	Follicular dendritic cells
FoxP3	Forkhead-box transcription factor P3
FVD	Fixable viability dye
GATA3	GATA binding protein 3

GCs	Germinal centers
HIV	Human immunodeficiency virus
HIV-1	HIV type 1
HIV-2	HIV type 2
HLA	Human Leukocite Antigens
ICOS	Inducible T-cell costimulator
ICOSL	ICOS ligand
IRF4	Interferon-regulatory factor 4
IL	Interleukin
IN	Integrase
IFNα	Interferon alpha
IS	Immune System
LTNPs	Long-term non-progressors
MA	Matrix (protein)
mAbs	Monoclonal antibodies
MFI	Median fluorescence intensity
MHC	Major Histocompatibility complex
mRNA	Messenger RNA
NC	Nucleocapsid (proteins)
NK	Natural killer
NRS	Normal rat serum
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PDL1	Programmed death ligand 1
Pha	Phytohaemagglutinin
PLOs	Primary lymphoid organs

PR	Protease
qPCR	Quantitative PCR
RM	Reaction mix
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RORγt	Retinoic acid receptor-related orphan receptor gamma
RT	Reverse transcriptase
RT-qPCR	Reverse transcription-quantitative PCR
SAP	SLAM – associated protein
SDB	Sample dilution buffer
SG-PERT	SYBR Green I-based product-enhanced reverse trancriptase
SHP1	SH2 domain-containing protein tyrosine phosphatase 1
SIDA	Síndrome da Imunodeficiência Adquirida
SIV	Simian Immune Deficiency Virus
SLAM	Signaling lymphocytic activation molecule
SLOs	Secondary lymphoid organs
STAT	Signal transducer and activator of transcription
SU	Surface (glycoproteins)
T-bet	T-box transcription factor
TCR	T-cell receptor
Tfh	Follicualr helper T follicular cell
Th	Helper T cell
TM	Transmembrane (glycoproteins)
TMNCs	Tonsil mononuclear cells
TNFα	Tumor necrosis factor alfa
Treg	T regulatory cell
VLB	Virus lysis buffer

Abstract

Acquired Immunodeficiency Syndrome (AIDS) is caused by two types of virus: HIV-1 or HIV-2. Much less attention has been given to HIV-2 infection because this epidemic is mostly confined to West Africa and some non-African countries, as is the case of Portugal. HIV-2 infection is characterized by a slow rate of CD4+ T cell loss and low levels of viral RNA in plasma, which clinically translates into a much longer asymptomatic phase and a slower progression rate AIDS, in comparison with infection by HIV-1. Recent studies have shown that follicular helper T cells (Tfh) represent largest main cell reservoir of HIV-1. These cells play a key role in the formation of the germinal centers in secondary lymphoid tissues, as well as in the subsequent selection, survival and differentiation of B cells with the ability to produce high-affinity antibodies. There are currently no data on Tfh in HIV-2 infection. The main objective of this study was to evaluate whether HIV-2 is able to infect Tfh. For this purpose, tonsils discarded during routine tonsillectomy were used as a source of secondary lymphoid tissue, and an in vitro assay of infection of purified cells was optimized. Different CD4 T cell subsets were infected with primary isolates of HIV-2 and HIV-1, using different co-receptors (CXCR4 or CCR5). For the first time, we showed that HIV-2 is able to infect Tfh, although the Tfh levels of proviral DNA were lower than those found upon HIV-1 infection performed in parallel. Moreover, Tfh were able to support viral replication, with an increase in total proviral DNA levels occurring 48 hours after stimulation of the infected cells and with evidence of non-defective viruses in the culture supernatants. Together, these results contribute to a better understanding of HIV-2 infection, revealing new insights into the ability of the virus to infect Tfh cells with new targets being identified for viral replication control.

Key-words: HIV-2 infection, follicular helper T cells (Tfh), human tonsils

RESUMO

A Síndrome da Imunodeficiência Adquirida (SIDA) é provocada por dois tipos de vírus: HIV-1 ou HIV-2. Tem sido dada muito menos atenção à infeção por HIV-2 por esta epidemia estar confinada maioritariamente à África Ocidental e a alguns países não-Africanos, como é o caso de Portugal. A infeção pelo HIV-2 caracteriza-se por um ritmo lento de perda de células T CD4 e baixos níveis de ARN viral no plasma. Clinicamente traduz-se numa longa fase assintomática e numa progressão mais lenta para o desenvolvimento de SIDA, relativamente à infeção por HIV-1. Estudos recentes têm vindo a demonstrar que as células T foliculares auxiliares (Tfh) representam um dos maiores reservatórios de HIV-1. Estas células têm um papel fundamental na formação dos centros germinativos dos tecidos linfoides secundários, bem como na subsequente seleção, sobrevivência, diferenciação de células B em células capazes de produzir anticorpos de alta afinidade. Este trabalho teve como principal objetivo avaliar a capacidade do HIV-2 para infetar as Tfh. Para isto, foi usado tecido linfoide de amígdalas retiradas por indicação clínica e otimizado um protocolo de infeção *in vitro* de células purificadas. Diferentes populações celulares de células T CD4 foram infetadas com isolados primários de HIV-2 e HIV-1, usando diferentes co-recetores (CXCR4 ou CCR5). Demonstrámos pela primeira vez que as Tfh são infetáveis pelo HIV-2, ainda que os níveis de DNA proviral total tenham sido inferiores aos obtidos nas infeções pelo HIV-1 efetuadas em paralelo. Mostrámos ainda que estas células são capazes de suportar a replicação viral, tendo-se verificado um aumento dos níveis de DNA proviral total, 48 horas após estimulação das células infetadas e comprovado a existência de vírus não-defetivos nos sobrenadantes das culturas. Em conclusão, estes resultados contribuem para o melhor entendimento da infeção por HIV-2, revelando novos conhecimentos sobre a capacidade do vírus infetar as células Tfh, tendo sido identificado novos alvos para o controle da replicação viral.

Palavras-chave: Infeção por HIV-2, células T foliculares auxiliares (Tfh), amígdalas humanas

1. INTRODUCTION

1.1. OVERVIEW OF THE IMMUNE RESPONSE TO INFECTIONS

One main function of the immune system is to protect the host from a universe of pathogens[1]. The mechanisms permitting their recognition and subsequent elimination can be broken down into two general categories: innate and adaptive responses.

The first line of defense is the innate immunity. The immune system acts rapidly, often from minutes to a few hours, providing a non-specific defense against infection and involving several cell types. Neutrophils, macrophages and dendritic cells (DC) are mainly responsible for the phagocytosis of microorganisms. Eosinophils are granulocytes that present phagocytic properties and play an important role in the destruction of parasites too large to be phagocytosed. Additionally, natural killer (NK) cells play an important role in the destruction of virus-infected cells, whereas mast cells and basophils initiate acute inflammatory responses mainly in the context of allergy[2,3].

On the other hand, adaptive immunity is a process that depends on the recognition of antigens and, thus, requires a Lag phase between the exposure to the antigen and the maximum response. This response is enhanced by the innate immunity. The hallmark of adaptive immunity is the generation memory. Therefore, upon re-encounter with the same antigen, the immune response is faster and more efficient. Cells of the adaptive immune system include T and B lymphocytes[4].

Regarding the organs constituting the immune system, they can be classified into two groups according to their function as primary or secondary lymphoid organs. Primary lymphoid organs comprise the thymus and bone marrow responsible for the production and maturation of T and B cell lineages, respectively. The secondary lymphoid organs are the main places where the immune responses are mounted[5] and include the lymph nodes, tonsils, adenoids, spleen, appendix, and Payer plaques[6]. Both T and B lymphocytes migrate from the primary lymphoid organs to the blood and the secondary lymphoid organs[7] through chemotactic gradients. They recirculate through the blood and secondary lymphoid organs to patrol for infections and when encounter

a pathogen they can interact effectively with the antigen, giving rise to antigen-specific immune responses in the secondary lymphoid organs[5,8].

T lymphocytes are activated through the interaction with antigen-presenting cells (APC), mainly DC as well as macrophages. These cells express major histocompatibility complex (MHC) proteins on their surface, which can be classified as MHC class I or MHC class II[9]. The interaction between the T cell receptor (TCR) and peptides presented by MHC class I molecules stimulates the differentiation of T cells into cytotoxic T lymphocytes, characterized by the expression of CD8 molecules on their surface[1]. Their clonal expansion produces effector cells capable of releasing substances that induce cellular lysis or apoptosis, promoting the elimination of neoplastic- or infected-cells[6]. Helper T (Th) cells, characterized by the expression of CD4 molecules, recognize MHC class II bound antigens[1]. They mediate the immune response by releasing cytokines that influence the activity and assist the performance of other cells[6]. After encountering an antigen in the periphery[10] and upon cell activation the immunologically naïve cells loose the expression of the CD45RA molecule, and start expressing the CD45RO molecule, typical of memory and effector T cells. Upon resolution of the infection, most CD8+ or CD4+ cells die by apoptosis and are phagocytosed. However, some are maintained as memory cells.

Another population of T cells, regulatory T lymphocytes (Treg), is responsible for suppressing, and therefore, controlling the immune responses[11,12]. A major subset of these cells express the Forkhead-box transcription factor P3 (FoxP3) which is considered the master regulator of their differentiation and function[12–14].

B cells, unlike previous ones, recognize free antigens through the B cell receptor (BCR)[15]. When activated, they proliferate and can differentiate either into memory or plasma cells. Memory B cells are found in peripheral blood and continue to express the receptor that binds to the antigen and are able to differentiate into plasma cells upon secondary encounters with their cognate antigen. Plasma cells are professional antibody producing cells that are stored in the bone marrow[1,15].

The CD4 T cell help, more precisely the help of a specialized subtype of Th cells, known as follicular T helper cells (Tfh)[16,17], is required to generate long-lived humoral responses.

1.2. FOLLICULAR HELPER T CELLS: ORIGIN, DIFFERENTIATION AND FUNCTION

The existence of Tfh cells was first reported in human secondary lymphoid tissue[18]. Tfh differentiate from naïve CD4 T cells through a complex and yet unclear process that involves multiple factors and consists of different steps[19].

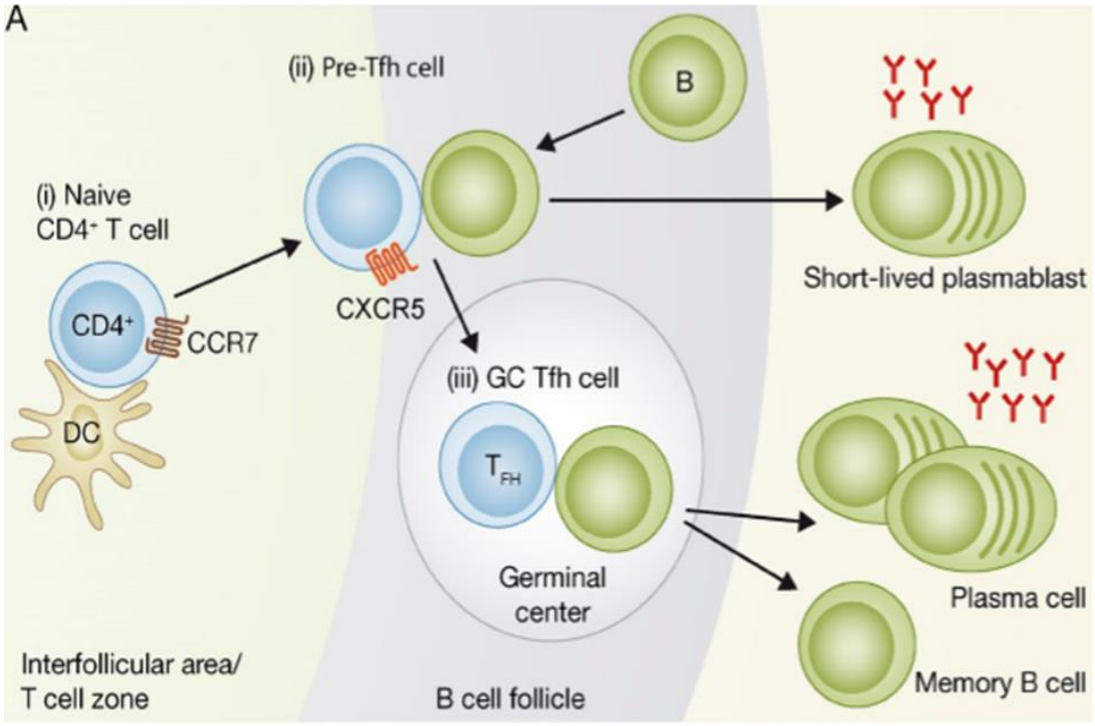

FIGURE 1 - TFH CELLS DIFFERENTIATION PROCESS. ***ADAPTED FROM MA, C. S. ET AL, JOURNAL OF EXPERIMENTAL MEDICINE (2012).*** Differentiation of Tfh is a highly regulated process, which begins in the T cell areas of the SLOs, where the naive Th cells are activated through the TCR after recognition of processed MHC II antigens presented by dendritic cell. This interaction provides signals that positively regulate the expression of the CXCR5 and negatively regulate expression of CCR7, allowing them to migrate to B cell follicles. Then, at the T-B border, these pre-Tfh interact with B cells, providing them help. This results in their differentiation into short-lived extrafollicular plasmablasts or their migration to follicles to form GCs, where Tfh continue to provide B-cell help, facilitating the generation of long-lived plasma cells and memory B cells.

Initial priming of CD4 T naïve cell by DC occurs in the T cell zone of secondary lymphoid tissues (Fig.4). Upon CD4 T cell stimulation, differentiation into Th1, Th2 or Th17 cells occurs. Afterwards, effector cells exit the lymphoid tissue and migrate to the site of infection or inflammation[20]. The generation of these effector T cell subsets depends on the stimulatory cytokines that are present in the microenvironment during

activation. The expression of IL-12, IL-4 and IL-6 or IL-23 promote the specific transcription factors T-box transcription factor (T-bet), GATA binding protein 3 (GATA3), and the retinoic acid receptor-related orphan receptor gamma (RORγt), in the case of Th1, Th2 and Th17 cells, respectively[21]. The differentiation of Tfh cells further requires the binding between inducible T-cell costimulatory (ICOS) and ICOS ligand (ICOSL). This interaction stimulates the expression of B cell lymphoma 6 (Bcl-6) factor and the consequent increase in transcription of the C-X-C chemokine receptor type 5 (CXCR5; homing receptor to B cell zone)[22].

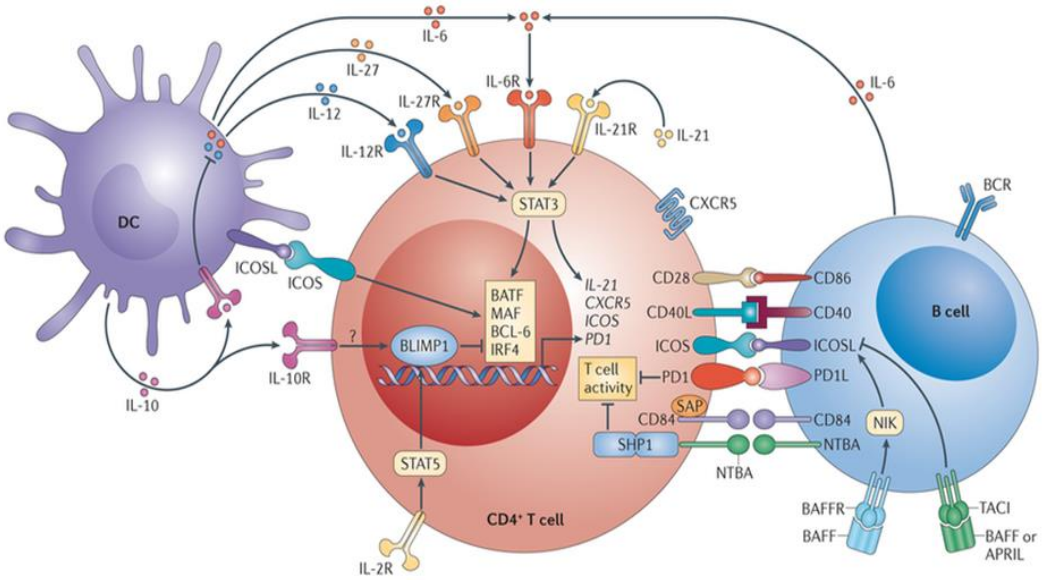

FIGURE 2 - TFH CELLS INTERACTIONS DURING DIFFERENTIATION. ***FROM TANGYE, S. G. ET AL, NATURE REVIEWS - IMMUNOLOGY (2013).*** Initial priming of naive CD4 T cells with DC requires the interaction of ICOS with its ligand, which induces the expression of CXCR5 and Bcl-6. Thereafter, DC produce IL-6, IL-12 and IL-27, which promotes the expression of different transcription factors. Simultaneously, the production of IL-21 by CD4 T cells, which acts autocrinously, also increases the expression of these transcription factors. These transcription factors induce the transcription of genes essential for the differentiation of Tfh, namely ICOS, PD-1, and again, CXCR5 and IL-21. Meanwhile, migration of pre-Tfh cells to T-B border mediated by the CXCR5 gradient, allows the selection and subsequent interactions with B cells. Interactions between CD28-CD86, CD40L-CD40 and ICOS-ICOSL are critical in this process.

Bcl-6 transcription factor is determinant for the commitment of the Tfh cell lineage and the increasing CXCR5 gradient stimulates the migration of pre-Tfh to the boundary between the T cell zone and the B cell follicle (T-B border)[23]. The CXCR5 increment is accompanied by a decrease in C-C chemokine receptor type 7 (CCR7), which is the receptor responsible for the entry of CD4+ cells into the T zone[22,24,25]. DC secrete a set of cytokines (IL-6, IL-12 and IL-27) which leads to an increase expression of transcription factors besides Bcl-6 via signal transducer and activator of transcription (STAT) 3 signaling. These are important regulatory functions during Tfh development, namely basic leucine zipper transcriptional factor ATF-like (BATF), MAF and interferon-regulatory factor 4 (IRF4) (Fig.2). Pre-Tfh releases IL-21, considered fundamental for Tfh[26], which can also act autocrinally. Increased expression of these factors not only contributes to increase CXCR5 and IL-21 expression, but also results in the transcription of genes essential for Tfh differentiation: ICOS and programmed death-1 (PD-1) [22,24,27].

At the T-B border, pre-Tfh selects a B cell that has a cognate antigen. CD28:CD86, CD40L:CD40, ICOS:ICOSL and PD-1:PD1 ligand 1 (PDL1) interactions are crucial for this step (Fig.5)[28]. B cells also send important signals for T cell maintenance, such as the release of IL-6. Other molecules are still involved in regulating the development of these cells. SLAM family receptors have double function in the formation of Tfh, and SAP protein stabilizes the interaction between T and B cells while SH2 domain-containing protein tyrosine phosphatase 1 (SHP1) suppresses it. In addition, Bcl-6 expression can be suppressed by the BLIMP1 protein, which is induced through the action of IL-2, in a STAT5-dependent manner[29].

The interaction of pre-Tfh with B cells can result in the production of short-lived extrafollicular plasmablasts; or in the migration of the pre-Tfh into the follicle, forming a structure called germinal center (GC)[18,30]. Within GCs occurs the selection of high affinity B cells and subsequent differentiation into memory B cells or plasma cells capable of producing highly specific antibodies (Fig.6). There are two mechanisms at the base of this process known as somatic hypermutation and immunoglobulin switching[20].

The selected B cells proliferate extensively in the dark zone (the denser zone of cells in the GC). Here, the BCR that represents the variable region of the immunoglobulin gene undergoes cycles of somatic hypermutation. Then, cells migrate to the light zone, where they are exposed to the antigen through follicular dendritic cells (FDC)[31]. B cells whose receptor has been mutated with higher specificity are selected by Tfh cells by receiving survival signals. Subsequently, they can re-enter the dark zone for a new cycle of somatic hypermutation, fine-tuning their specificity or differentiating into memory B cells and plasma cells[32]. The remaining B cells undergo cell death by apoptosis.

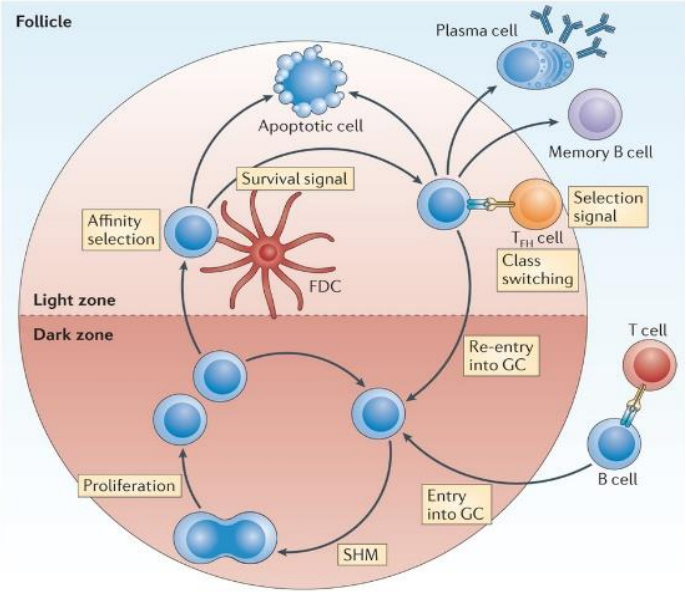

FIGURE 3 - GERMINAL CENTER REACTION. ***FROM HEESTERS, B. A. ET AL, NATURE REVIEWS - IMMUNOLOGY (2014)****.* The reaction that occurs at the germinal center and allows the selection of high affinity B cells, followed by differentiation into memory B cells and plasma cells is due to a phenomenon known as somatic hypermutation. In this process FDC are responsible for the exposure of the antigen to B cells, after one or more cycles of somatic hypermutation. Cells with poor affinity for the antigen undergo apoptosis. The interaction of Tfh with B cells, whose receptor was mutated is of the greatest importance, since Tfh are responsible for the switching of immunoglobulins.

Tfh are also essential to induce the class switch recombination of immunoglobulins.

Tfh cells play a major role in the formation and maintenance of GCs, but also in the selection and promotion of highly specific B cell survival and differentiation into memory B cells and plasma cells[33,34].

Therefore, Tfh play a key role in the responses to infections and to the development of vaccines[20]. As described below Tfh have recently been shown to be a preferential target of HIV-1 infection[35].

1.3. THE HUMAN IMMUNODEFICIENCY VIRUS

The human immunodeficiency virus (HIV) belongs to the *Lentivirus* genus and integrates the *Retroviridae* family[36]. It is the etiological agent of the acquired immunodeficiency syndrome (AIDS), a disease characterized by the progressive deterioration of the immune system. This condition allows the development of opportunistic infections and potentially deadly cancers[37].

Simian immune deficiency virus (SIV) is considered to have been the ancestor of HIV and transmission to humans has derived from separate non-human primates, HIV-1 from SIVcpz and HIV-2 from SIVsmm[38].

1.3.1. HIV/AIDS EPIDEMICS

AIDS is one of the most devastating epidemics in human history, having killed about 35 million people. It remains a leading cause of death worldwide and the first cause of death in Africa[39]. HIV-1 is responsible for pandemics globally, while HIV-2 causes epidemics mainly located in West Africa. HIV-2 has also been detected in non-African countries, most of which with historical and socio-economic ties to West Africa, where this virus is endemic. Portugal is considered the European country with the highest prevalence of HIV-2 infection[40,41]. In 2006, HIV-2 was responsible for 5.4% of AIDS cases in Portugal[40]. In 2008, about 1813 cases of HIV-2 infection were notified[41].

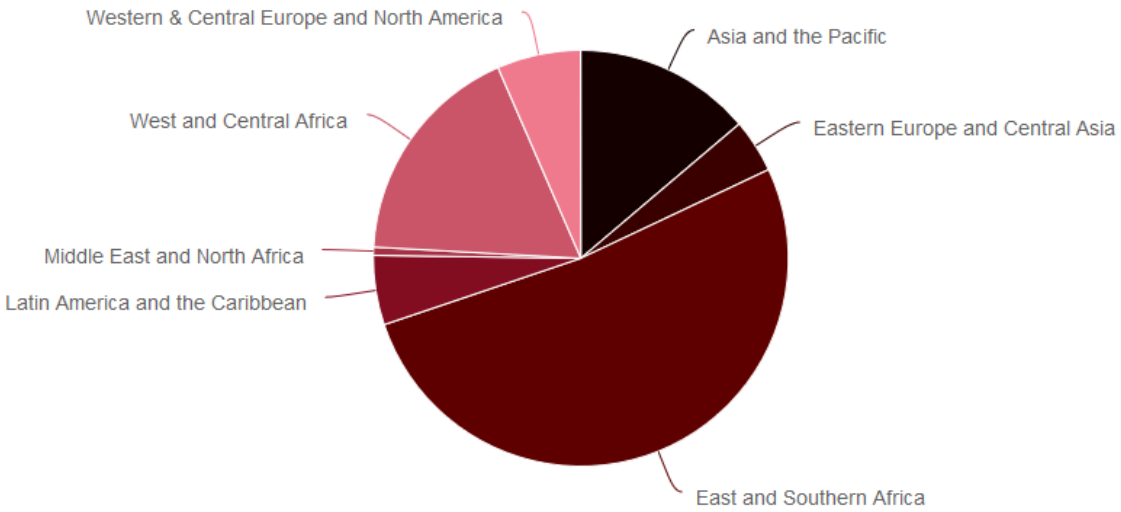

FIGURE 4 - PEOPLE LIVING WITH HIV (ALL AGES) BY REGION. ***FROM UNAIDS ESTIMATES (2016).***

According to the latest UNAIDS data covering 160 countries[42], by 2015 there were 36.7 million people living with HIV (Fig.3), compared with 33.3 million in 2010. This may be a result of new infections, people living longer with HIV and overall population growth. Partly due to the increase in ART, a 45% decrease of AIDS-related deaths occurred, reducing deaths to 1.1 million people.

In the same year, there were about 2.1 million new infections, which represents 5700 new infections per day, most of them transmitted heterosexually. Women represent half of all adults living with HIV worldwide; young people (ages between 15 and 24) account for approximately a third of new HIV infections, and globally, there were 1.8 million children living with HIV. Although they may seem scary, these data show the huge improvement already made and what can be achieved in the upcoming years with a commitment to end the AIDS epidemic by 2030[43].

1.3.2. STRUCTURAL AND GENOMIC ORGANIZATION

HIV-2 and HIV-1 are similar in morphological and genomic organization terms. Still, they differ 55% in their nucleotide sequences[44].

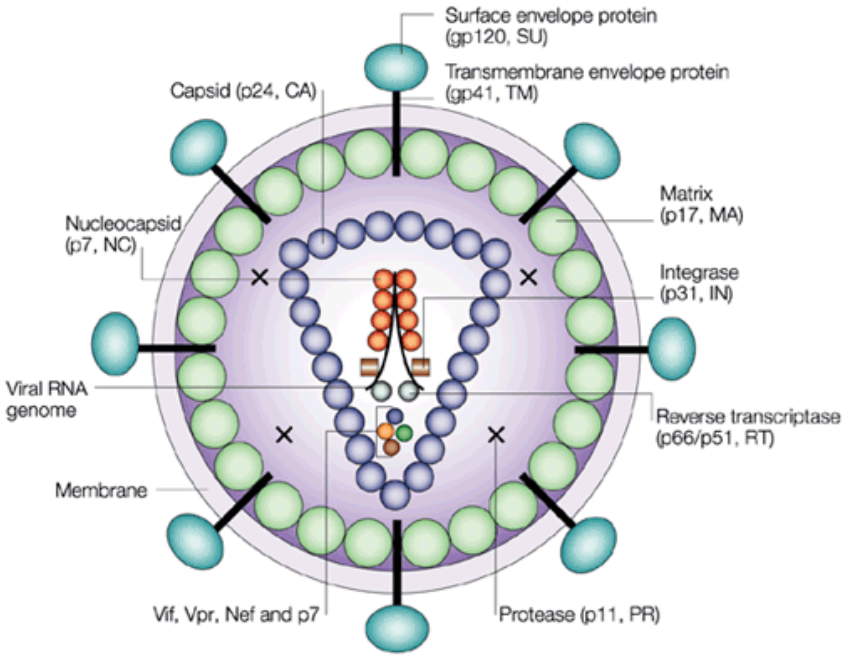

FIGURE 5 - STRUCTURE OF HIV-1. ***ADAPTED FROM ROBINSON, H. L., NATURE REVIEWS - IMMUNOLOGY (2002).***

Regarding morphology, the mature viral particles present 10 nm diameter and a lipid bilayer membrane coat, usually derived from the host cell (Fig.5)[45].

Concerning genomic organization, there is a high degree of genetic diversity between the two lentiviruses, particularly in their *env* genes[46,47]. The same gene can produce proteins of different sizes and distinct cleavage sites, depending on the type of virus. This latter particularity may be relevant for the design of drugs[48].

The *env* genes of HIV-2 and HIV-1 encode the precursor 140 and 160 kDa glycoproteins (gp140 and gp160, accordingly). These precursors are cleaved by a host protease into the HIV-2 125 kDa and HIV-1 120 kDa surface (SU) glycoproteins (gp125 and gp120) and the 36 and 41 kDa transmembrane (TM) glycoproteins (gp36 and gp41) for HIV-2 and HIV-1, respectively[49]. Besides, it may incorporate different host cell proteins such as MHC class I and MHC class II proteins.

Internally, the viral particle is coated by the matrix protein (MA) (p16 and p17 for HIV-2 and HIV-1), which is essential for virion integrity. The viral capsid (CA), consisting of the p26 protein in HIV-2 and p24 in HIV-1, contains two identical ribonucleic acid

(RNA) molecules associated with nucleocapsid proteins (NC): p6 and p7 for HIV-2 and HIV-1[50]. These structural proteins are encoded by the *gag* gene, which also encodes the C-terminal protein p6 in case of HIV-2 and p7 of HIV-1.

HIV also presents all the enzymes necessary for replication, such as the reverse transcriptase (RT) p53 in HIV-2 and p66/p51 dimeric complex in HIV-1 virions; the protease (PR/p11 for both viruses) and integrase (IN) (p34 in HIV-2 and p31 in HIV-1)) encoded by the *pol* gene[51].

Besides, the HIV genome contains genes that encode two regulatory proteins (Tat and Rev) and four accessory proteins (Nef, Vif, Vpr and Vpx in HIV-2 or Vpu in HIV-1)[52].

1.3.3. VIRUS LIFE CYCLE

HIV infection (Fig.6) generally begins with a membrane fusion mechanism common to both viruses[53]. The virus binds to the CD4-specific receptor, essentially present on the cell surface of Th, monocytes, macrophages and DC. In addition to CD4, there are chemokine receptors expressed on the cell surface that are also essential for the interaction between the virus and the host cell. In vivo, the major co-receptors for HIV are CCR5 and CXCR4[54], which are differentially expressed on CD4 T cells and determine cell tropism[55].

Binding of the SU glycoprotein to the host cell CD4 receptor causes changes in glycoprotein conformation by exposing the co-receptor binding domain. In turn, this binding induces a rearrangement of the TM glycoprotein, allowing the fusion of the envelope with the cell membrane, and consequently, entry of the viral nucleocapsid into the cell[56,57].

In the cytoplasm, RT performs reverse transcription of viral RNA into double-stranded DNA after uncoating the viral capsid. By this time, formation of the pre-integration complex occurs, which also includes IN, MA and Vpr viral proteins, responsible for transporting the complex to the nucleus.

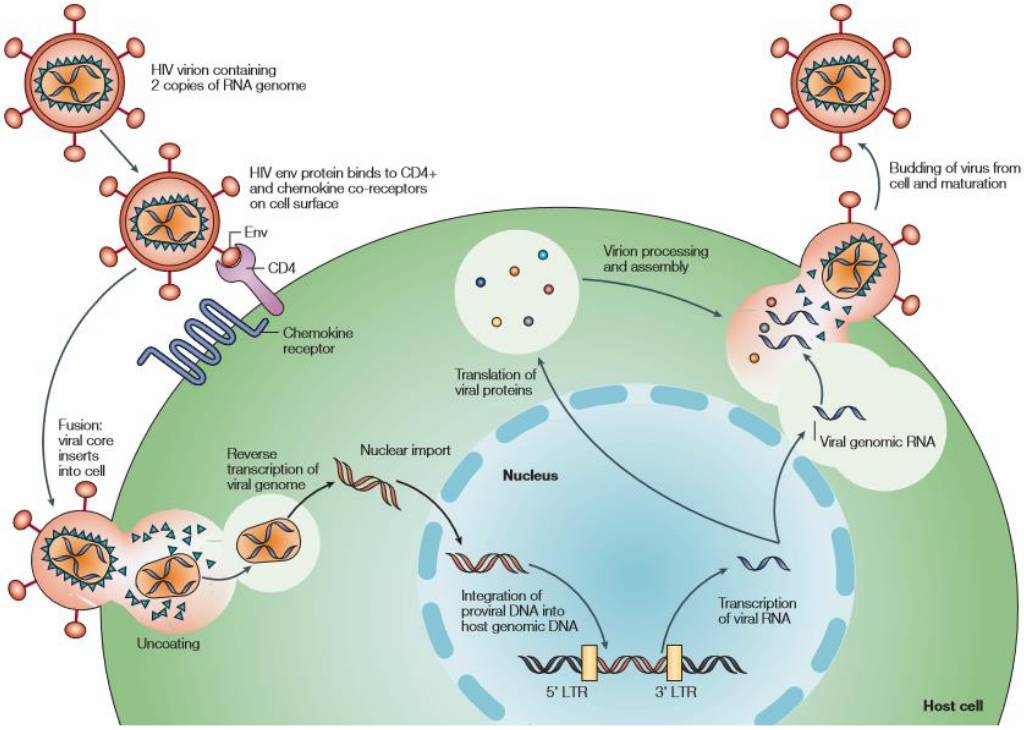

FIGURE 6 - REPLICATION CYCLE OF HIV. ***FROM RAMBAUT, A. ET AL, NATURE REVIEWS - GENETICS (2004).*** Initially, the virus binds to the CD4 receptor and co-receptors, after conformational changes. Fusion occurs and the virion contents are released into the cell. Once in the cytoplasm, the uncoating of viral capsid allows the reverse transcription of the viral RNA. HIV can integrate the host DNA and use the machinery to produce new viruse or remain latent.

Once in the nucleus, IN integrates the viral DNA into the genome of the host cell, where it persists in the latency state[53]. Alternatively, integrated proviral DNA progresses to viral replication once the host cell is activated using the cellular transcriptional machinery[58].

The RNA and the viral proteins produced translocate to the cell surface, where new immature viral particles are formed. New viruses are released along with a lipid bilayer from the host cell. Final maturation occurs with the cleavage of the Gag-Pol precursor protein by PR. Subsequently, rearrangement and structural positioning of the viral proteins occur, producing mature and infectious viral particles[59].

1.3.4. HIV-2 VS HIV-1 INFECTIONS

HIV-2 and HIV-1 are very similar at their basic gene arrangement, intracellular replication pathways and modes of transmission (sexual contact, perinatal transmission and blood-borne exposure). Both viruses cause immune failure and AIDS[60]. However, despite sharing the same spectrum of clinical manifestations, the impact of HIV-2 and HIV-1 infection are quite different. HIV-2 infection presents a better prognosis and usually a much slower disease progression[60–62]. The HIV-2 infected individuals generally present a clinical latency period of 10 years or more. Some may never progress to AIDS and even during the symptomatic phase, HIV-2-infected patients present a survival time greater than patients with HIV-1 AIDS[63].

The rate of disease progression can be influenced by both host and viral determinants (i.e. viral load and viral phenotype)[64]. Nevertheless, usually the time from initial infection to the development of AIDS among HIV-1-untreated patients ranges from 8 to 10 years. A typical pattern of HIV-1 infection is characterized by the acute infection, asymptomatic phase and symptomatic phase[65]. The acute infection starts upon viral exposure and may be associated in around half of the patients with clinical manifestations similar to those observed in infectious mononucleosis (fever, pharyngitis, fatigue and other)[66]. The cellular and humoral responses against HIV-1 typically develop within the first two to four weeks, and lead to the decline of plasma viral load, with an establishment of an equilibrium between the viral and the host. During the asymptomatic phase there is a progressive loss of CD4 T cells and disruption of the lymphoid organs that culminate in the AIDS period. In the absence of antiretroviral therapy, death usually occurs within two years of AIDS.

A major hallmark of HIV infection is the chronic immune activation, which promotes viral replication and CD4 T cell depletion. Immune activation is characterized by elevated plasma levels of proinflammatory cytokines, such as interferon alpha (IFNα), tumor necrosis factor alpha (TNFα), interleukin 6 (IL-6), IL-1 and IL-18. These environment can contribute to additional immune activation and apoptosis of immune cells[67].

Despite the prolonged asymptomatic phase among HIV-2-infected patients, lack of treatment also results in a CD4 cell count decline. Subsequently, these individuals become vulnerable to the same range of opportunistic infections and develop illnesses similar to HIV-1-patients[61]. Nevertheless, in contrast to HIV-1, CD4 depletion typically occurs at a much slower rate in HIV-2 infection. HIV-2-infected individuals are considered to be attenuated disease models and they can be referred to as long-term non-progressors (LTNPs) or elite controllers (ECs). These nomenclatures take into account the fact that in the absence of antiretroviral therapy (ART), most of these patients are able to maintain high CD4 T cell counts over many years (LTNPs) or show viral loads below the limit of detection for more than two years (ECs)[47,68]. Notably, plasma viral load is very low or undetectable in untreated HIV-2 patients, even if the proviral DNA levels are similar to those found in HIV-1-infections[69–71].

Sousa, A. E. *et al.* (2002) showed that CD4 T cell depletion is directly related to immune activation in both HIV-2 and HIV-1 infections, although the viral load in the plasma of HIV-2-infected patients is two orders of magnitude lower than in HIV-1 patients[73].

Soares, R. S. *et al.* (2011) found that untreated HIV-1- and HIV-2-infected individuals, matched for CD4 T cell depletion, exhibited similar Gag mRNA levels, suggesting that significant viral transcription is occurring in untreated HIV-2 patients, despite the reduced viremia. Tat mRNA transcripts were also assessed since they are thought to be expressed mainly either in recently infected cells or cells actively producing virus. At last, significantly lower levels in HIV-2 patients were observed, suggesting that the rate of *de novo* infection is decreased in these patients[72].

The HIV-2 infectivity rate of both vertical and horizontal transmissions is also lower, according to the reduced plasma viremia[49], even though an explanation remains unclear. The differential production of host restriction factors, which integrates a group of intrinsic cellular defense mechanisms against viruses, may be at the origin of viral replication inhibition[74]. Moreover, it is observed the production of neutralizing antibodies during the clinical latency phase, contrary to what happens during HIV-1 infection[47]. These antibodies are capable of recognizing various HIV strains and inhibiting their ability to infect during the chronic phase of HIV-2 infection[75].

Nevertheless, Tendeiro, R. et al has revealed that the infection with HIV-2 unexpectedly results in the decrease of B cells[76].

Another feature of the immune activation is the increased T cell turnover, followed by a loss of T cell regenerative capacity[48]. Sousa, A. E. *et al.* (2002) evaluated T cell turnover in HIV-2- and HIV-1-infected individuals with similar CD4 T cell counts but different viral loads. This analysis was performed by assessing the expression of Ki67 nuclear factor, which is upregulated in all cell cycle phases except G_0[78]. It was shown an increase in CD4 T cells expressing Ki67, similar in both infections, indicating that CD4 depletion is more related to the overall activation and turnover of T cells than to the rate of virus replication[77].

HIV mainly infects activated CD4 T cells[79], where viral replication is rapid and efficient[80]. In agreement, memory cells comprise a greater portion of latent proviral infection than do naïve cells[81]. A significant correlation between the frequency of CCR5-expressing cells within the CD4 subset and the degree of CD4 depletion was observed with an HIV-2 cohort but not with HIV-1. Importantly, CCR5 expression was largely confined to the memory CD4 T cell compartment[82].

Regarding the treatment, HIV-2 is intrinsically resistant to some commonly used classes of antiretroviral drugs, usually effective against HIV-1[61].

The explanation behind why viruses present distinct natural histories is not known[83,84]. Therefore, a comparison between these two infections is extremely pertinent for hypothesis-generation and hypothesis-testing.

1.4. ROLE OF FOLLICULAR HELPER T CELLS IN HIV INFECTION

During the initial phases of HIV-1 infection, there is an increase in Tfh cell expansion[85] and several studies showed GCs as the major reservoir for HIV-1[86–88]. It has been suggested that viral infection occurs mainly at these sites, with viral replication occurring in Tfh cells[89,90]. On average, 60-75% of HIV-1-producing cells are within B cell follicles, and CD4 T cells at these sites are 40 times more prone to be infected than a cell located in another compartment [91,92]. Besides, in HIV patients, Tfh cells were found to

contain the highest percentage of CD4 T cells carrying HIV DNA and were the most efficient in supporting productive infection *in* vitro[92].

Virus preference for Tfh cells characteristically leads to an abnormal humoral immune response, and consequent loss of memory B cells and plasma cells[93,94]. Cubas *et al* (2013) demonstrated that inadequate Tfh cell help impaired B cell responses during HIV infection, contributing to B cell selection and maturation failure[95–97].

Several factors predispose these Tfh cells to high levels of viral infection and replication: expression of central memory phenotype[85]; expression of CXCR4 and CCR5 coreceptors[35]; presence of a network of FDC with high amounts of associated virions, adjacent to Tfh[98]; reduced penetration of ART drugs into GCs[99]; and lack of cytotoxic T cells in GCs[91,100,101], although recent studies have focused on the role of CXCR5-expressing CD8 T cells. Nevertheless, the mechanisms that promote HIV-1 replication in Tfh are not fully understood.

There are no data on the ability of HIV-2 to infect Tfh.

2. OBJECTIVES

Numerous studies have been performed on HIV-1, while much less attention has been given to HIV-2.

Although HIV-2 infection is potentially fatal, LTNPs prevail in abundance. This dichotomy is one of the most intriguing dogma regarding HIV topic. Revealing the underlying mechanisms of HIV-2 infection becomes crucial both to a better understanding of HIV-1 infection as to the identification of essential biomedical targets in the design of novel therapeutic strategies.

In the present study, we ask whether HIV-2 is able to infect the Tfh subset. Ultimately, we expect that our results contribute to better understand the role of Tfh cells in HIV infection and to define strategies to harness Tfh cells to control HIV.

The following work aims to complement the scarce knowledge that currently exists regarding HIV-2 infection using different human primary tonsillar CD4 lymphocytes populations, with special attention given to Tfh cells. Importantly, infections were performed with different primary isolates of HIV-2, but also of HIV-1 in parallel. Specifically, this project intends to assess the infection and replication capacities of HIV-2 as compared to HIV-1. The analysis involved extensive flow cytometry and quantitative PCR (qPCR). To the author's knowledge, this subject was never studied before, contributing to the clarification of some questions in an innovative way.

3. METHODOLOGY

3.1. HUMAN SAMPLES

Buffy coats from healthy donors were provided by the Instituto Português do Sangue e da Transplantação (Lisbon, Portugal) after written informed consent. Tonsil specimens were obtained through routine pediatric tonsillectomy at the Hospital Santa Maria (Lisbon, Portugal) after written informed consent by parents or legal representatives. These samples would be otherwise discarded.

3.2. GENERATION OF VIRAL STOCKS USING PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS)

PBMCs were used to produce viral stocks for further experiments.

3.2.1. ISOLATION OF PBMCS

PBMCs were isolated from buffy coats of different healthy donors, through a Ficoll-Paque Plus (GE Healthcare) density gradient. Briefly, 15mL of blood were diluted in the same volume of PBS, and then 15mL of Ficoll-Paque solution were added to the bottom of the tube. After 20 minutes of centrifugation at 2000 rpm, the resulting ring containing the PBMCs was placed in a 50mL falcon. Cells were washed twice with PBS by centrifuging at 1500 rpm for 15 minutes and then at 1200 rpm for 10 minutes. Next, cells were diluted in acetic acid to exclude erythrocytes and counted using a Neubauer chamber. PBMCs were cultured in RPMI (Rosewell Park Memorial Institute (Gibco) supplemented with 10% of fetal bovine serum (FBS), 2mM of L-glutamine (L-Glu), 100U/mL of Penicilin/Streptomycin and 50µg/mL of Gentamicin (Complete medium; CM) at density of a $2x10^6$ cells per mL, and stimulated with 5µg/mL of phytohaemagglutinin (PHA; Sigma) at 37°C and 5% CO_2 for 3 days. PBMCs were then maintained for a period of 10 to 12 days in CM supplemented with 10U/mL of IL-2 (Maurice Gately, Hoffmann-La Roche Inc., through the NIH AIDS reagent Program).

3.2.2. INFECTION OF PBMCS

Five million PBMCs were incubated with CM plus IL-2 at 10U/mL and 3µg/mL polybrene (Sigma) along with 1 mL of highly concentrated viruses for three hours, in a BioSafety Laboratory Level 3 (BSL3) Facility. Subsequently, CM plus IL-2 at 10U/mL and 3µg/mL polybrene (Sigma) was added and PBMCs were cultured at $2x10^6$ cells/mL, at 37°C and 5%CO_2. At day one of infection $10x10^6$ PBMCs were added to each culture. Two thirds of the supernatant were collected every three days, stored at -80°C and replaced through the addition of $20x10^6$ PBMCs. At the end of the 21-day culture, all supernatants stored throughout production were thawed, pooled and divided into 1 mL aliquots for storage at -80°C.

3.2.3. QUANTIFICATION OF PRODUCED VIRUSES BY SYBR GREEN PRODUCT-ENHANCED RT (SG-PERT)

Viruses were quantified by measuring RT activity using the SG-PERT assay. Virus lysis was performed at the BSL3 by addition of 5µL of each virus supernatant to 5µL of 2X virus lysis buffer (VLB) (0.25% Triton X-100 (Sigma), 50mM potassium chloride (KCl; Sigma), 100mM Tris-chloride (Tris-HCl) buffer pH 7.4 (Sigma), 40% Glycerol (Sigma) and DNA/RNAse free water (Sigma)), containing 2µL of Ribolock RNAse inhibitor (Fermentas). After a 10-minute incubation at room temperature, samples were diluted by adding 90µL of 1X sample dilution buffer (SDB) (50mM ammonium sulfate [$(NH_4)_2SO_4$); Sigma), 200mM KCl (Sigma), 200mM Tris-HCl buffer pH8.3 (Sigma) and DNA/RNAse free water (Sigma)).

For the real time PCR reaction, in a 96-well plate, 10µL of lysed virus were added to 10µL of 2X reaction mix (RM) (2X SDB, 10mM magnesium chloride ($MgCl_2$; Sigma), 0.2mg/mL bovine serum albumin (BSA; Isaza), 400µM dATP (Invitrogen), 400µM dGTP (Invitrogen), 400µM dTTP (Invitrogen), 400µM dCTP (Invitrogen), 1µM forward primer (5'- TCCTGCTCAACTTCCTGTCGAG-3'; Invitrogen), 1µM reverse primer (5'-CACAGGTCAAACCTCCTAGGAATG-3'; Invitrogen), 7pmol/mL MS2 RNA (Roche), 1:10 000 SYBR Green I (Invitrogen) and DNA/RNAse free water (Sigma)), containing 5U of TrueStart HotStart Taq DNA (Thermo Scientific). In parallel, a standard curve using defined RT concentrations (5000pg/mL, 1000pg/mL, 200pg/mL, 40pg/mL, 8pg/mL,

1.6pg/mL and 0.32pg/mL) was run. Negative controls included the RM plus water, as well as SDB complemented with RM and VLB. The reaction was performed at ABI 7500 Fast (ThermoFisher Scientific) using the following protocol: 30 minutes at 33ºC; 2 minutes at 95ºC; 40 Cycles of 5 seconds at 95ºC, 5 seconds at 60ºC, 15 seconds at 72ºC, 25 seconds at 82ºC (acquisition); and melting curve.

3.2.4. ASSESSMENT OF VIRAL STOCKS' INFECTIVITY

Infectivity of viruses produced was confirmed by infecting activated PBMCs purified from the blood of three different volunteer healthy donors together. PBMCs ($3.5x10^6$ cells) were infected with 1050pg of RT of each virus. Viruses were centrifuged at 50.000 rpm for 30 minutes, the supernatant removed and ressuspended in 200µL of CM plus IL-2 at 10U/mL and 3µg/mL polybrene (Sigma) and added to cells previously distributed in 500µL of CM plus IL-2 at 10U/mL and 3µg/mL polybrene (Sigma) in a 48-well plate. Cells were incubated at 37ºC and 5%CO_2 for 4 hours and then washed with PBS and ressuspended in CM plus IL-2 at 10U/mL and 3µg/mL polybrene (Sigma) at $2.0x10^6$ cells/mL. Finally, infected cells were distributed in two 24-well plates and were incubated for 7 days at 37ºC and 5%CO_2. PBMCs in CM plus IL-2 at 10U/mL and 3µg/mL polybrene (Sigma) only were used as negative control. DNA samples were extracted as described in section RNA/DNA purification and total viral DNA was quantified as described in TOTAL VIRAL DNA QUANTIFICATION section.

3.3. ISOLATION AND CULTURE OF TONSIL MONONUCLEAR CELLS (TMNCS)

Tonsil specimens were processed by removing blood and cauterized parts, using tweezers and scissors, cut in smaller pieces and mashed over a 70µm filter into one or two (depending on the tonsil size) 50mL falcon with RPMI (Rosewell Park Memorial Institute (Gibco) supplemented with 2% of fetal bovine serum (FBS) (RPMI2). Then, 10 mL of Ficoll-Paque solution were added to 30 mL of cell suspension and TMNCs were isolated through a Ficoll-Paque Plus (GE Healthcare) density gradient. After 15 minutes of centrifugation at 2100 rpm, the resulting ring containing the TMNCs was collected and washed twice with RPMI2. Next, cells were diluted in acetic acid to exclude

erythrocytes, counted using a Neubauer chamber and subsequently ressuspended in freezing medium (FBS supplemented with 20% Dimethyl sulfoxide (DMSO, Sigma)) before storage at -80^{o}C.

Culture of TMNCs in 96-well round bottom plates was performed with complete medium for cells (RPMI1640 supplemented with 10% FBS, 2mM L-Glutamin, 100U/mL Penicillin/ Streptomycin, 1X non-essential aminoacids, 1mM sodium pyruvate and 50µg/mL Gentamicin [all from Gibco]; CM_{cells}) in three different cell densities ($10x10^6$, $5x10^6$ and $2.5x10^6$ cells per mL). During a period ranging from four to seven days, the cell viability and phenotypic characteristics of TMNCs were assessed everyday through flow cytometry.

3.4. FLOW CYTOMETRY

Surface staining was performed for 30 minutes at room temperature and always included FVD for dead cell exclusion. In case of intracellular staining, cells were fixed, permeabilized and stained using an intracellular staining kit (eBioscience) according to the manufacturer's instructions. Monoclonal antibodies (mAbs) used are listed below.

TABLE 1 - LIST OF ANTIBODIES USED IN THIS STUDY.

Molecule	*Clone*	*Fluorochrome*	*Brand*
Bcl-6	K112-91	eFluor 647	BD Biosciences
CCR5	2D7/CCR5 (RUO)	APC – Cy7	BD Biosciences
CCR6	R6H1	PE – Cy7	eBioscience
CD3	OKT3	FITC	eBioscience
CD3	OKT3	eFluor 450	eBioscience
CD3	UCHT1	V500	BD Biosciences
CD4	RPA-T4	BV – 711	BioLegend
CD8α	RPA-T8	PE – Cy 7	eBioscience
CD45RO	UCHL1	FITC	eBioscience

CD57	TBO1	eFluor 450	eBioscience
CD57	TBO1	FITC	eBioscience
CXCR4	12G5	APC	eBioscience
CXCR5	J252D4	PE	BioLegend
FoxP3	PCH101	eFluor 450	BioLegend
ICOS	C398.4A	PerCP Cy 5.5	BioLegend
Ki67	B56	PerCP Cy 5.5	BD Biosciences
PD-1	EH12.2H7	BV – 605	BioLegend

3.5. CD4 T CELL SORTING

Frozen TMNCs were thawed at the day before the sorting. Cells were collected in RPMI supplemented with 20% FBS (RPMI20), centrifuged five minutes at 1600 rpm, ressuspended in CM_{cells}, counted with trypan blue and cultured overnight in $10cm^3$ Petri dishes, at $10x10^6$ cells per mL.

Total TMNCs were washed with PBS, by centrifuging for five minutes at 1600rpm. Afterwards, surface staining was performed for 30 minutes at room temperature, using the anti-human mAbs listed in Table 1. FVD was always included for dead cell exclusion. Next, cells were washed with PBS supplemented with 2% FBS for five minutes at 1600 rpm and ressuspended in PBS supplemented with 2% FBS. Cell subpopulations were sorted in FACS Aria IIu cell sorter (BD Biosciences) and recovered in FACS tubes with cap containing CM_{cells}.

In order to optimize the sorting of CXCR5, ICOS and PD-1 subsets, a pre-sorting enrichment was performed. For this purpose, purified untouched CD4+ T cells were obtained from total TMNCs, using a Human CD4 T Cell Isolation Kit – MojoSort™ Magnetic Cell Separation System (Cat No. 480010, BioLegend), according to manufacturers' instructions. The subsequent sample preparation procedure was similar to the one described above, with the exception that a third marker was used (see RESULTS for gate strategy) and instead of PBS supplemented with 2% FBS, it was used PBS supplemented with 10% of FBS.

3.6. INFECTION OF TMNCS AND CELL SUBPOPULATIONS

HIV infection was performed in a BSL3 facility. Viruses were centrifuged at 50.000rpm for 30 minutes. Next, they were ressuspended in CM_{cells} plus 3 µg/mL polybrene at concentrations indicated below and added to each cell culture (medium only as negative control).

For TMNCs infection, five million cells were infected with three different concentrations (0.4, 0.8 or 1.6 ng/10^6cells) of X4-tropic HIV-1 primary isolate (92HT599). After three hours, cells were washed with PBS and cultured in a 24-well plate for 24 hours with CM_{cells} plus 3 µg/mL polybrene, at $5x10^6$ cells per mL, 37ºC and 5%CO_2. After this, cells pellets and supernatants were collected and stored at -80ºC.

For cell subsets infection, 2.5 $x10^5$ cells of each population were infected with 1.6 ng per 10^6cells of R5- or X4-tropic HIV-2 (R5-tropic viruses 60415K and HCC6.03+; X4-tropic viruses 20.04 and HSM10) and HIV-1 primary isolates (R5-tropic 92HT660 and X4-tropic 92HT599), in a 96-well plate. After 3 hours, cells were washed with PBS and cultured at $2x10^6$ cells/mL for 24h with CM_{cells} plus 3 µg/mL polybrene, at 37ºC and 5%CO_2. After 24 hours, cells were transferred to a new 96-well plate previously coated with 1µg/mL of α-CD3 (eBioscience) and cultured with 1µg/mL of α-CD28 (eBioscience). At this time, approximately 50.000 cells were collected, washed and stored as dry pellets at -80ºC. After 48 hours of stimulation, both cells and supernatants were collected and stored at -80ºC.

3.6.1. RNA/DNA PURIFICATION

RNA and DNA were extracted from cell pellets of infected TMNCs or cell subsets, using the ZR-Duet DNA/RNA MiniPrep kit (Zymo research), according to manufacturers' instructions. This kit allows the extraction of both DNA and RNA. The DNA was stored at -20ºC and the RNA was stored at -80ºC.

3.6.2. TOTAL PROVIRAL DNA QUANTIFICATION

Total proviral DNA was quantified using quantitative real-time PCR (qPCR). Two sets of primers previously designed and validated in the lab were used. In order to quantify total proviral DNA in number of gag copies per million cells, both gag and CD3 were amplified in the same sample. In parallel, standard curves containing a known number of copies (10^7 to 5 copies/5µL) were run using plasmids containing both HIV-1 or HIV-2 gag and CD3. Table 5 and Table 6 show the primers, probes, PCR reaction mix and PCR conditions used in the process. Reactions contained 1X TaqMan® Gene Expression Master Mix (Applied Biosystems), 400 nM each primer (Table 2), 250 nM probe (Table 2) and 50ng 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).

TABLE 2 - PRIMERS AND PROBES USED IN RT-PCR TO QUANTIFY TOTAL PROVIRAL DNA.

Primers and Probes

HIV-1 gag	*Primer Fw*	5'-CGAGAGCGTCAGTATTAAGC-3'
	Primer Rv	5'-AGCTCCCTGCTTGCCCATAC-3'
	Probe:	5'-FAM-CCCTGGCCTTAACCGAATT-MGB-3'
HIV-2 gag	*Primer Fw*	5'-CGCGAGAAACTCCGTCTTG-3'
	Primer Rv	5'GCTGCCCACACAATATGTTTTA-3'
	Probe:	5'- FAM-CCGGGCCGTAACCT-MGB-3'
CD3	*Primer Fw*	5'-AGGGCAAAATGGAGGCTCTTA-3'
	Primer Rv	5'-TCTCCTCCATGGGACACTGTT-3'
	Probe:	5'- VIC-CTCTCTAGCAGAGAAGAGT-MGB-3'

3.6.3. VIRAL INFECTIVITY ASSESSMENT

TZM-bl cell line was used to assess the virus infectivity, through the chlorophenol red-β-D-galactopyranoside (CPRG) assay.

3.6.4. CULTURE OF TZM-BL

Cells were cultured in T75 culture flasks, grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% FBS, 100U/mL PenStrep and 2mM L-glutamine. Cells were kept in sterile conditions at 37°C and at a partial pressure of 5% CO_2. Every three to four days, cells were splitted into a new culture flask. For this purpose, cells were washed with 1mL of TrypLE™ Express (Gibco). Then, it was added 2mL of TrypLE™ Express (Gibco) and cells were incubated for two to five minutes at 37ºC. Trypsinized cells were ressuspended in complete DMEM and centrifuged five minutes at 1600 rpm. After that, cells were ressuspended in 10mL of medium and prepared in new flasks, according to the dilution needed (to split cells within three to four days, a dilution 1:20 was performed; within 2 days, a dilution 1:10 was performed).

3.6.5. CHLOROPHENOL RED-β-D-GALACTOPYRANOSIDE (CPRG) ASSAY

To evaluate if the different cell subpopulations were able to support productive infection, the virus infectivity of the supernatants of the culture of stimulated cells was determined, as a measure of β-*galactosidase* expression by Chlorophenol red-β-D-galactopyranoside (CPRG) colorimetric assay, according to the manufacturers' recommendation (Roche, Germany) and as described by Mammano *et al.* (2000). For this purpose, TZM-Bl cells containing the β-gal gene under the control of HIV-LTR were used. On the day before infection, TZM-Bl cells were seeded at 3.5 x 10^4 cells per well in 96-well plate flat bottom. Twenty-four hours later, viral supernatants from infected sorted populations were thawed, and 40 µL of each supernatant was used to infect seeded TZM-Bl cells. After 3-4 hours of incubation at 37°C, all supernatants were removed and 200 µL of complete DMEM were added to each well. Forty hours post-infection, cells were washed with PBS and then lysed with CPRG lysis buffer ($MgCl_2$ 5mM + NP40 0.5% in PBS) before adding the CPRG substrate. Optical densities were read at 570 nm, with a reference filter set at 690 nm, in Infinite M200 plate reader (Tecan).

4. RESULTS

4.1. PROTOCOL OPTIMIZATION

4.1.1. PRODUCTION OF HIV-2 AND HIV-1 PRIMARY ISOLATES

PBMCs were used to generate viral stocks of primary HIV isolates. The usage of primary isolates has a major advantage, since they are genetically closer to the viruses isolated from infected individuals. Although lab-adapted strains are mainly used, their long-term use including in cell lines is associated with viral changes[102]. The primary isolates were selected because we wanted to find out if the use of different co-receptors would result in distinct infection read-outs. The 92HT599 X4-tropic and 92US660 R5-tropic HIV-1 viruses and 20.04 X4-tropic and 60415K R5-tropic HIV-2 viruses were already produced. To complement the existing stocks[70], we produced the HSM10 X4-tropic and HCC6.03+ R5-tropic HIV-viruses (Table 3).

After 21 days of culture, the amount of each virus produced was measured by SG-PERT assay (Table 3).

TABLE 3 – VIRAL STOCKS PRODUCED AND VIRAL QUANTIFICATION BY RT ACTIVITY USING SG-PERT.

Primary Isolates		*Co-receptor usage*	*Quantity mean (pg/µL)*
HIV-2	HSM10	X4	2243.6
	HCC6.03+	R5	3330.1

4.1.2. VIRAL TITER

In order to determine the best infection conditions, TMNCs were infected with 3 different concentrations (0.4, 0.8 or 1.6 ng/10^6cells) of the X4-tropic HIV-1 virus (92HT599).

The infection was confirmed by qPCR quantification of the number of HIV-1 gag copies produced per million of cells. As Table 4 shows, there was a dose-response curve

with all concentrations working well. We chose to use 1.6 ng/10^6cells in the following experiments, because the number of *gag* copies obtain in this condition allows a better discrimination which is crucial in a short period infection as performed. Of note, the hypothesis of using a higher concentration of virus was discarded due to unbearable viral manipulation in BSL3 facility.

TABLE 4 – TOTAL VIRAL DNA LEVELS OF TMNCS.

Virus concentration (ng/10^6 cells)	***gag* copies/10^6 cells**
0.4	860
0.8	1680
1.6	2740

4.1.3. CELL CULTURE CONDITIONS

4.1.3.1. CELL DENSITY

In order to define the optimal culture conditions, TMNCs were cultured in three different cell densities ($10x10^6$, $5x10^6$ and $2.5x10^6$ cells per mL). It was verified that the density of $5x10^6$ cells/mL was associated with the best cell viability in culture. However, given the limited number of cells from each subset obtained after cell sorting, the cell density used in experiments performed with cell populations was $2x10^6$ cells/mL.

4.1.3.2. COMPARISON OF FRESH AND FROZEN CELLS

Next, we evaluated whether the profile of frozen cells would be different from that observed when using fresh TMNCs. As already done by other authors, the profile was defined considering the expression of PD-1 and CXCR5 markers. Cell subsets were identified as Tfh (CXCR5+ PD1+ cells), double-negative (CXCR5- PD1- cells; DN), Pre-Tfh (CXCR5+ PD1low cells) and "Activated" (CXCR5- PD1+ cells). As can be observed in the following dotplots (Figure 7), populations from frozen TMNCs remained well defined. These findings supported the use of frozen cells, in agreement to other several studies[95,103–105].

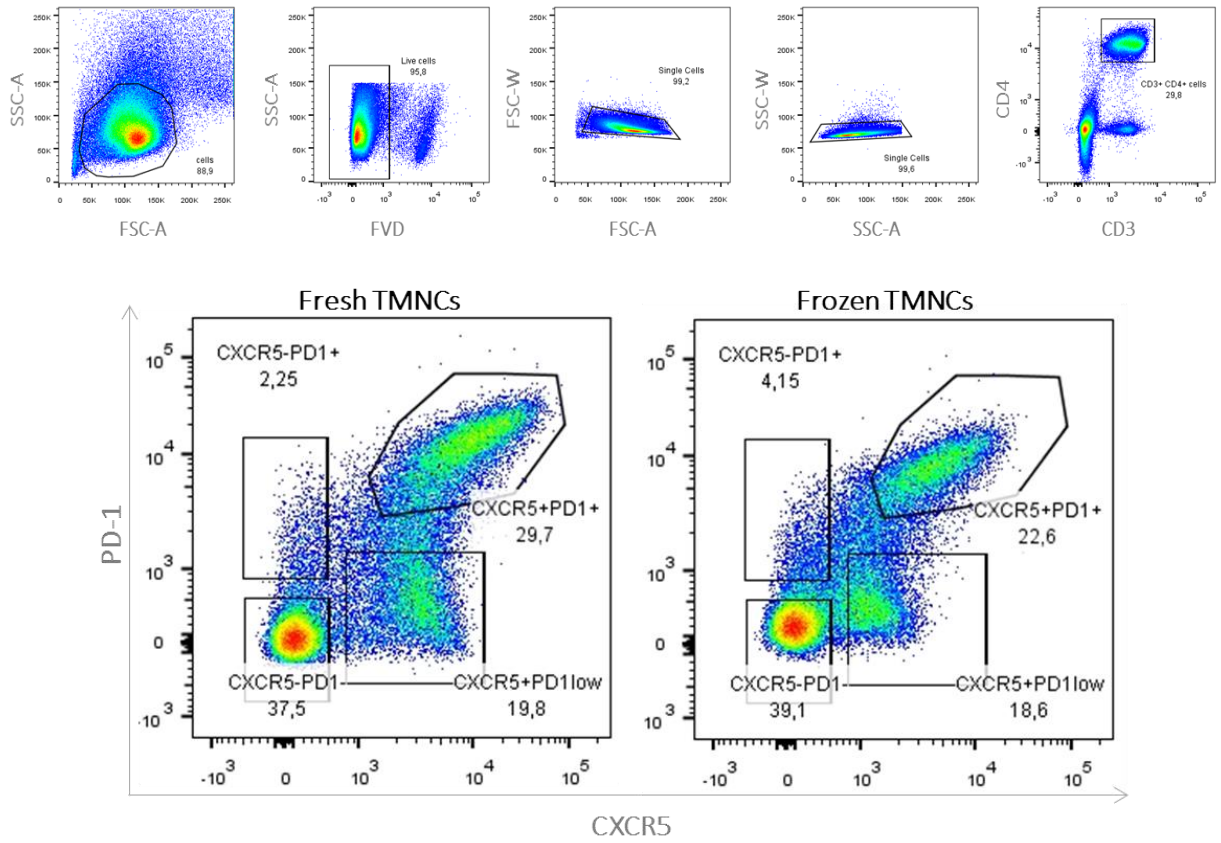

FIGURE 7 - POPULATION DISTRIBUTION OF FROZEN TMNCS APPEAR TO BE COMPARABLE TO THOSE OF FRESH TMNCS. Fresh TMNCs were isolated and immediately stained with mAbs and FVD. Frozen TMNCs underwent the freezing process for at least 7 days. After thawing, they were stained with respective mAbs and FVD. Cells were acquired in an LSRFortessa cell analyzer (BD Biosciences) and data were analyzed by FlowJo Software (TreeStar). Gate strategy is also depicted.

4.1.3.3. DYNAMICS OF TMNCS IN CULTURE

After defining the best culture conditions and verifying that frozen TMNCs could be used, the changes of phenotype of TMNCs during the culture period were studied. This study would help to determine the time that cells could be kept in culture without altering their phenotypic characteristics and, more important, without losing the definition of the populations that we aim to evaluate at the end of the culture. For this purpose, TMNCs were cultured for several days (maximum 7 days), considering the culture parameters defined previously, and cells were collected every day for phenotype analysis, as well as to determine cell viability.

Tfh population (which is the population of most interest) drastically lost expression of the markers that defines it, shortly after 24 hours of culture (Figure 8.A). Concerning

cell viability, a slight increase in the percentage of dead cells over time could be observed (Figure 8.B), as expected.

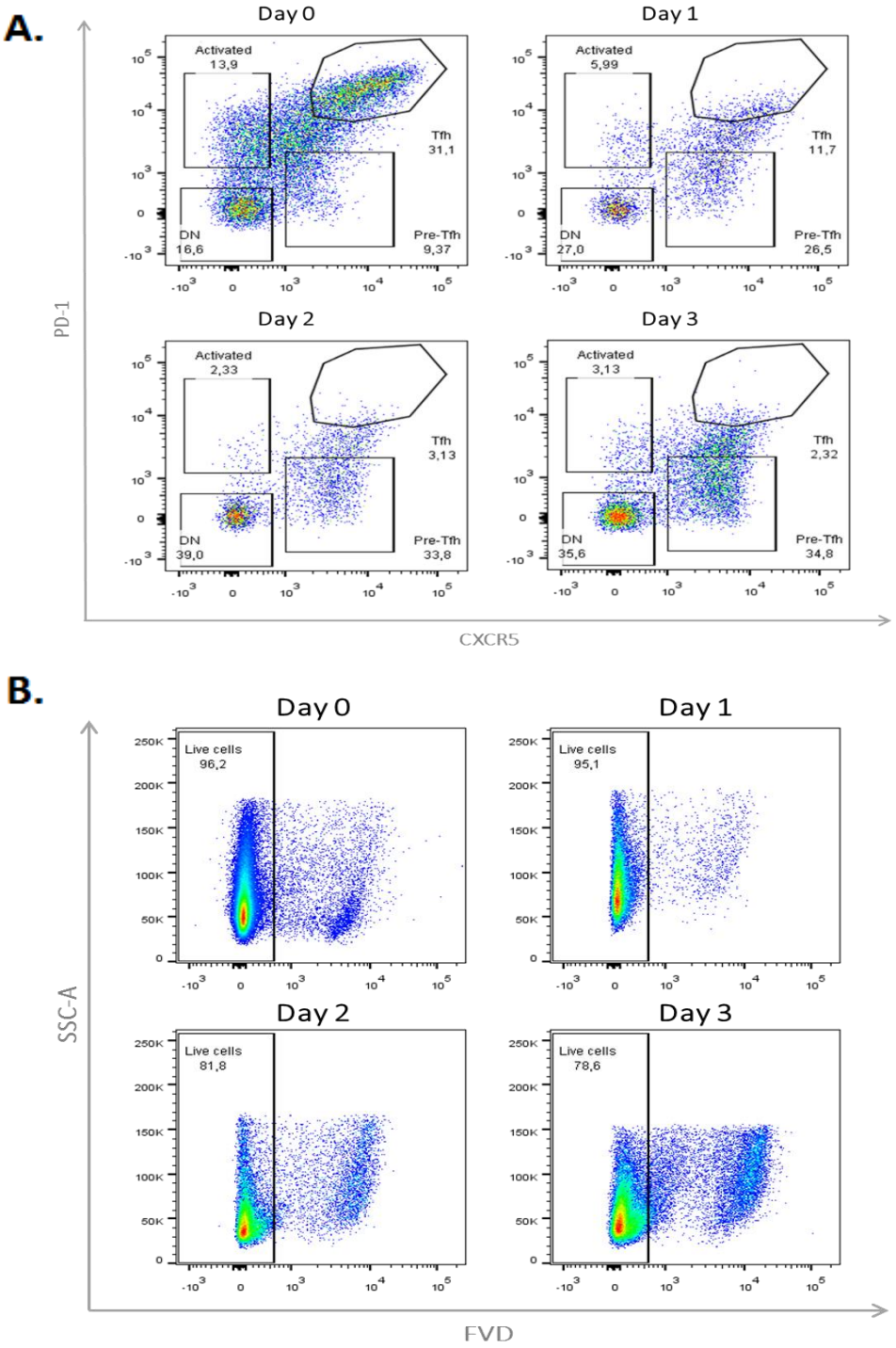

FIGURE 8 - TMNCS PHENOTYPE CHANGES DURING CULTURE. TMNCs were cultured at $5x10^6$ cells/mL and collected and stained each day with mAbs to characterization of CD4 T cell subsets (A) and FVD to assess cell viability (B). Cells were acquired in a LSRFortessa cell analyzer (BD Biosciences) and data was analyzed by FlowJo Software (TreeStar). Gate strategy employed was the same as of Figure 8. Comparison of day 0 up to day 3 are shown. Representative data of one out of four experiments using different tonsils are depicted.

In conclusion, the use of prolonged cultures was excluded, as well as the study of the cell phenotype after infection, since the culture alone affects the expression of these markers.

4.1.3.4. CULTURE OF PURIFIED CD4 T CELL SUBSETS FROM THE TONSILS

In parallel, the changes observed upon culture of the purified four main CD4 T cell populations were also analyzed. It is important to note that when culturing TMNCs, the CD4 subsets interact not only with each other, but also with other cell types, namely monocytes. Thus, the culture environment of the purified sub-populations is entirely different from culturing TMNCs, with possible impact on their survival and phenotype.
The following CD4+ T cell subsets Tfh (CXCR5+ PD1+), DN (CXCR5- PD1-), Pre-Tfh (CXCR5+ PD1low) and "Activated" (CXCR5- PD1+) were purified from TMNCs, cultured for three/four days, and expression of the major markers and cell viability was evaluated. As illustrated in Figure 9, our data suggest that Tfh cells progressively lost the expression of CXCR5 and PD-1; there no major alterations in the DN and pre-Tfh populations; and with respect to "Activated" cells, there was a tendency to increase the expression of CXCR5.

Regarding cell viability, Tfh were the most sensitive subset, with a considerable increase in dead cells already on day 2 of culture (Figure 10). The same tendency occurred in the "Activated" subset, although the increase in the percentage of dead cells throughout the culture is not as marked (Figure 10). Pre-Tfh and DN showed no major impairment in survival over the 3 days of culture (Figure 10).
Since the culture alone affects the expression of PD-1 and CXCR5 markers, prolonged cultures of purified CD4 T cell subsets were also excluded, as well as the study of the phenotype of the cells after infection.

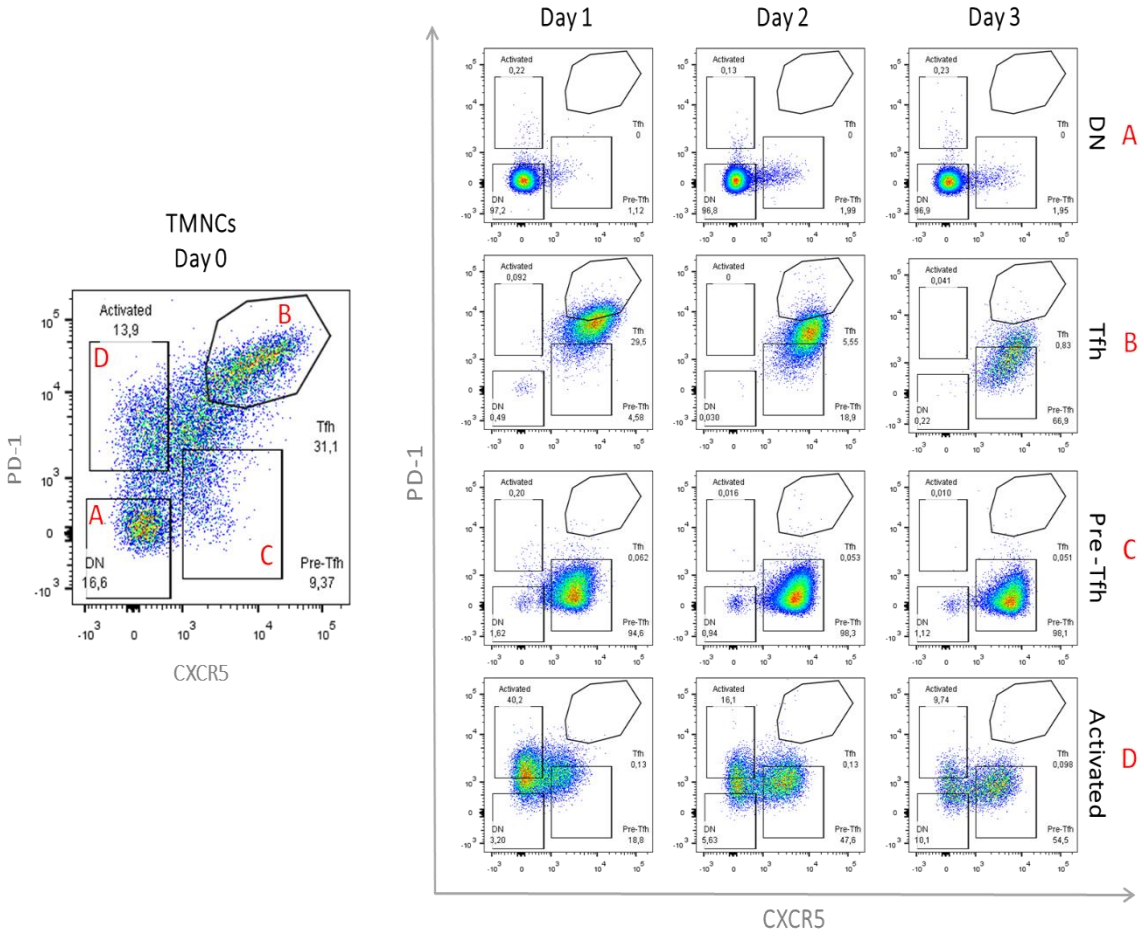

FIGURE 9 - CELL SUBSET PHENOTYPE CHANGES DURING CULTURE. Cell subsets were cultured in CM_{cells} at $2x10^6$cells/mL, for a maximum of 4 days. Everyday cells were collected and stained with mAbs and FVD. Cells were acquired in a LSRFortessa cell analyzer (BD Biosciences) and data was analyzed by FlowJo Software (TreeStar). Gate strategy to analyze the cell phenotype was the same presented in Figure 8. Representative data of one out of four experiments.

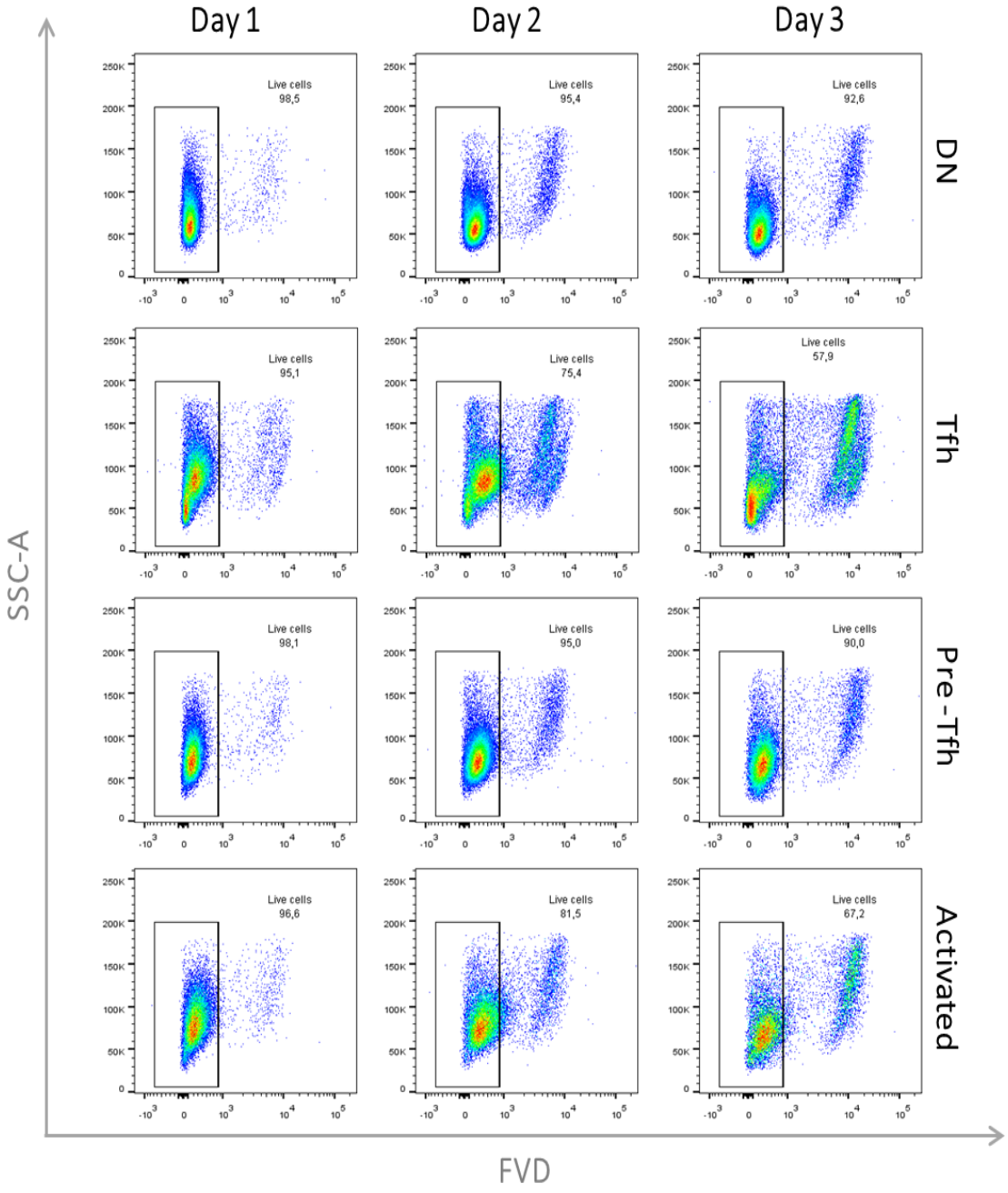

FIGURE 10 - CELL SUBSET VIABILITY DURING CULTURE. Cell subsets were cultured in CM_{cells} at $2x10^6$ cells/mL, for a maximum of 4 days. Everyday cells were collected and stained with mAbs and FVD. Cells were acquired in a LSRFortessa cell analyzer (BD Biosciences) and data was analyzed by FlowJo Software (TreeStar). Gate strategy to analyze cell viability was the same presented in Figure 8. Representative data of one out of four experiments.

4.1.4. CHARACTERIZATION OF THE CD4+ T CELL SUBSETS OF INTEREST

In addition, we further characterized the CD4+ T cell subsets from the tonsil to validate our gating strategy, through the expression of different surface and intracellular cellular markers.

Expression of Bcl-6 transcription factor was evaluated in all populations. As expected, Tfh showed the highest expression of this key regulator, thus confirming that our definition of Tfh through PD-1 and CXCR5 is valid (Figure 11).

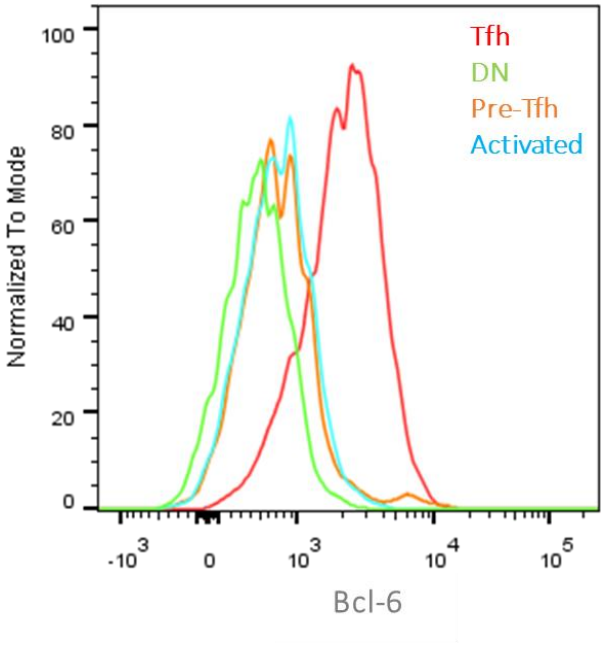

FIGURE 11 - TFH CELLS EXPRESS HIGHER LEVELS OF BCL-6 RELATIVE TO OTHER CELL POPULATIONS. The expression of Bcl-6 was assessed within Tfh, DN, pre-Tfh and activated cell subsets. Cells were acquired in a LSRFortessa cell analyzer (BD Biosciences) and data was analyzed by FlowJo Software (TreeStar). Gate strategy was the same presented in Figure 8. Representative data of one out of four experiments.

Since viruses with different coreceptors usage were used, the expression of CXCR4 and CCR5 was observed, as illustrated for Tfh and DN populations in Figure 12. High expression of CXCR4 could be observed in Tfh cells, contrary to lower expression in DN subset. Regarding CCR5, the expression was similar in both populations.

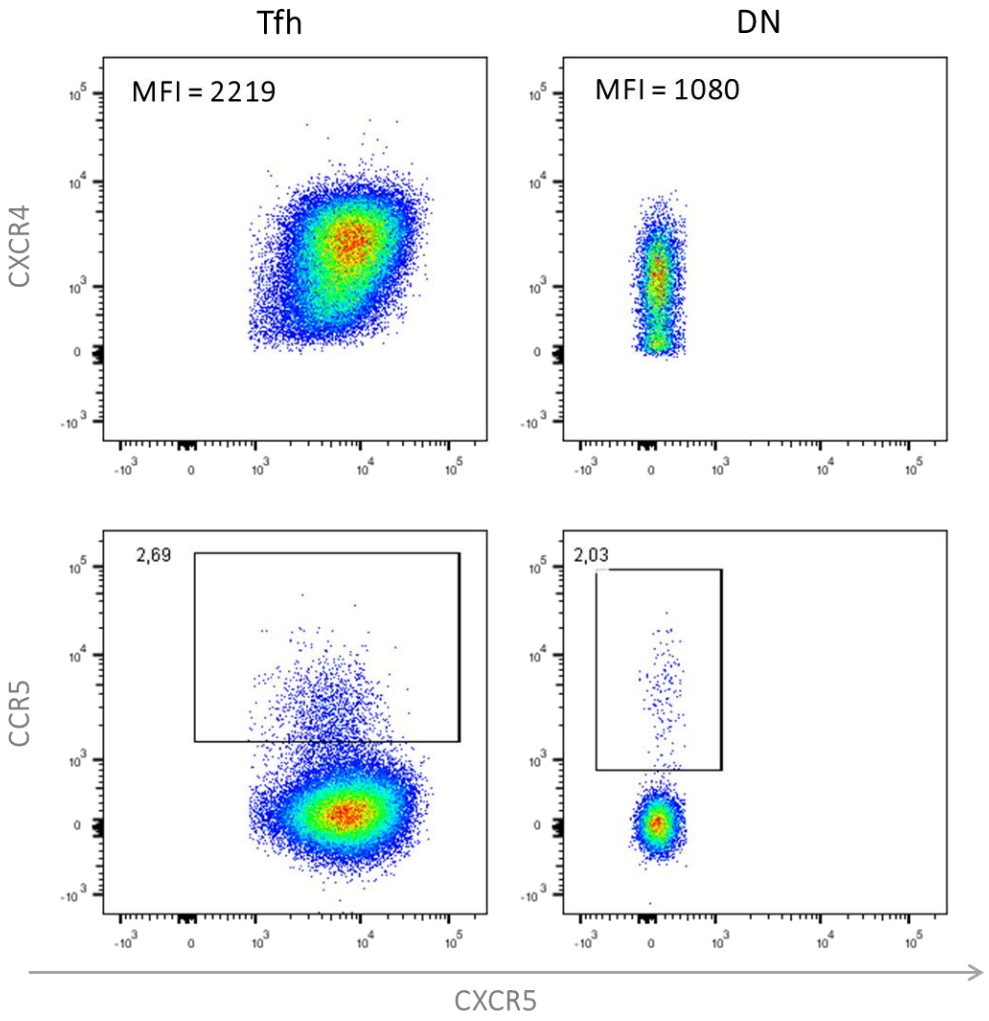

FIGURE 12 - EXPRESSION LEVELS OF CXCR4 AND CCR5 CORECEPTORS. The expression of CXCR4 and CCR5 were assessed within Tfh and DN cell subsets. Cells were acquired in a LSRFortessa cell analyzer (BD Biosciences) and data was analyzed by FlowJo Software (TreeStar). Gate strategy was the same presented in Figure 8. Representative data of one out of four experiments.

The expression of CD57 activation marker, as well as of CD45RO memory marker, was also assessed (Figure 13). CD57, also a marker of germinal centers[104], was particularly found in Tfh cells, contrary to what was seen in the case of DN. Concerning

CD45RO, most of Tfh were found to express this antigen and about 9% of DN cells also presented this molecule, which is in agreement with their phenotype in terms of PD-1 and CXCR5 markers.

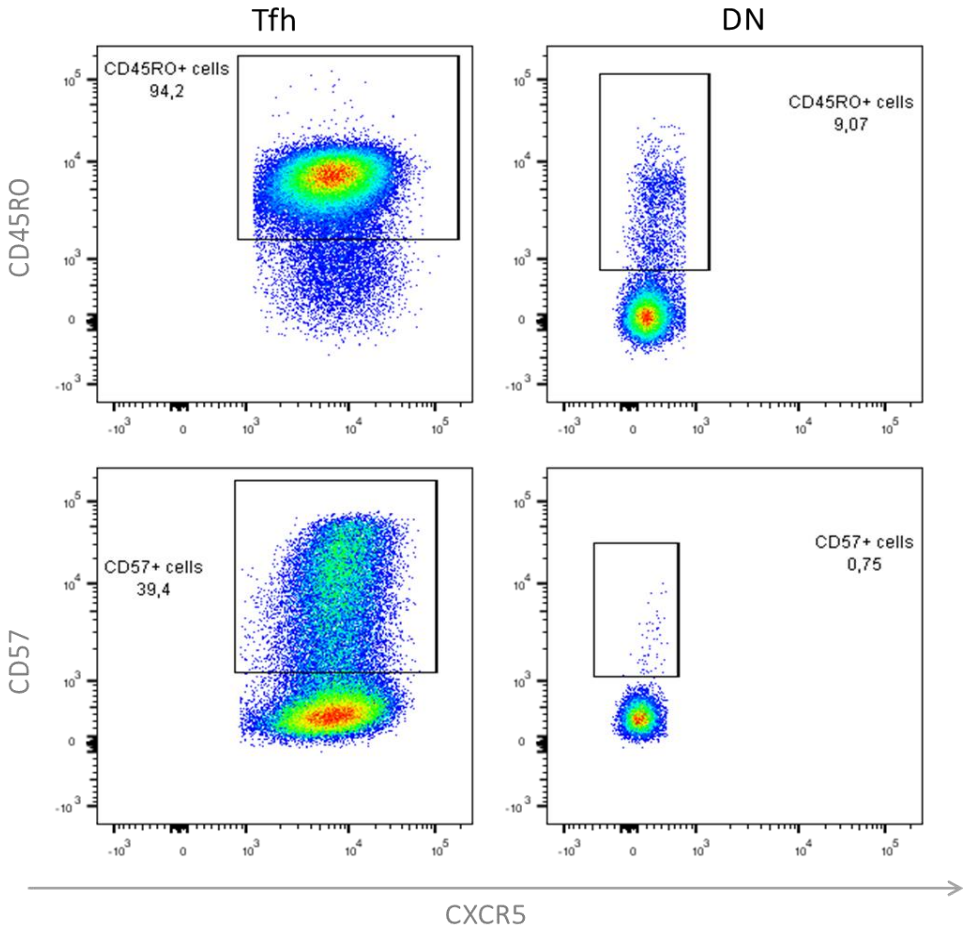

FIGURE 13 - EXPRESSION LEVELS OF CD45RO AND CD57 MARKERS. The expression of CD45RO and CD57 were assessed within Tfh and DN cell subsets. Cells were acquired in a LSRFortessa cell analyzer (BD Biosciences) and data was analyzed by FlowJo Software (TreeStar). Gate strategy was the same presented in Figure 8. Representative data of one out of four experiments.

Because expression of the CD45RO memory marker was observed in DN cells, we decided to analyse the ICOS levels in the different populations (Figure 14). Since this molecule is typical of Tfh, it could eventually be used to define populations in addition to CXCR5 and PD-1. All populations revealed the expression of ICOS, with special attention to the DN population, since over 50% of the population expressed this marker.

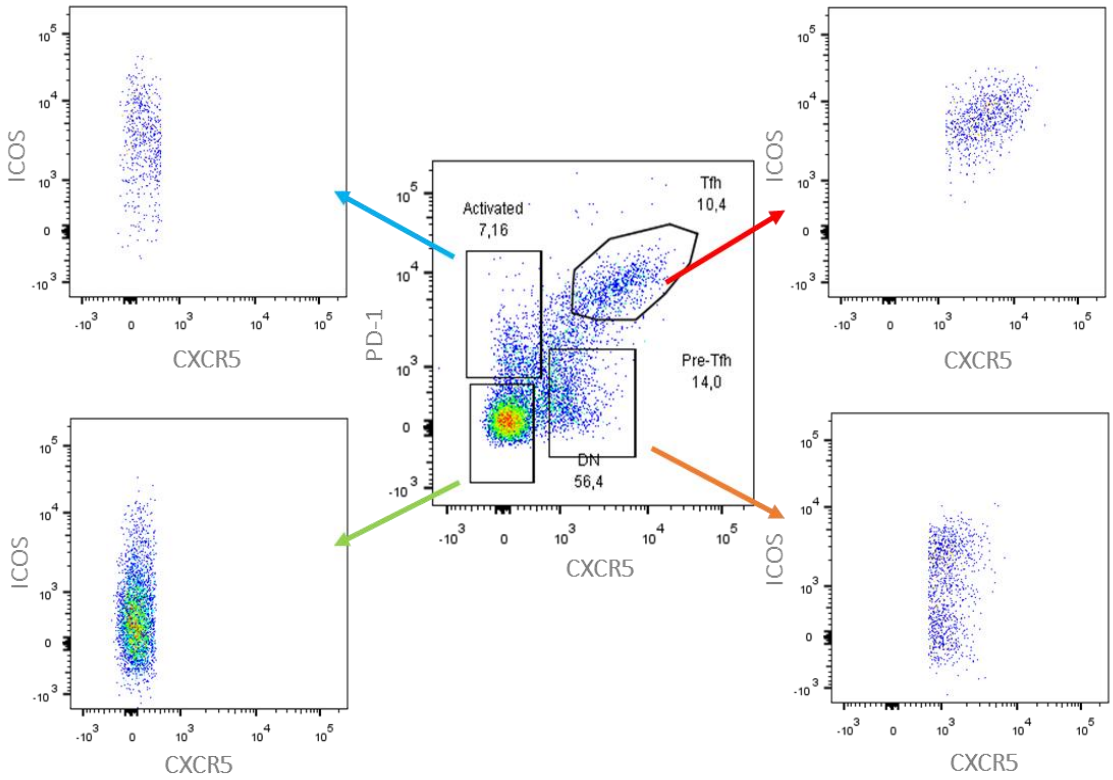

FIGURE 14 - ALL SUBPOPULATIONS EXPRESS ICOS. The expression of ICOS was assessed within Tfh, DN, pre-Tfh and activated cell subsets. Cells were acquired in a LSRFortessa cell analyzer (BD Biosciences) and data was analyzed by FlowJo Software (TreeStar). Gate strategy was the same presented in Figure 8.

4.1.5. SORTING STRATEGY

Since the expression of ICOS was verified in all populations, including DN cells that we expected to express a naïve-like phenotype, a new sorting strategy was chosen to better define the populations. Thus, Tfh cells were defined as $CXCR5^{+}$ $PD1^{+}$ $ICOS^{+}$, DN as $CXCR5^{-}$ $PD1^{-}$ $ICOS^{-}$, pre-Tfh as $CXCR5^{+}$ $PD1^{-}$ $ICOS^{+/-}$ and "Activated" as $CXCR5^{-}$ $PD1^{+/-}$ $ICOS^{+/-}$. The sorting strategy is shown in Figure 15.

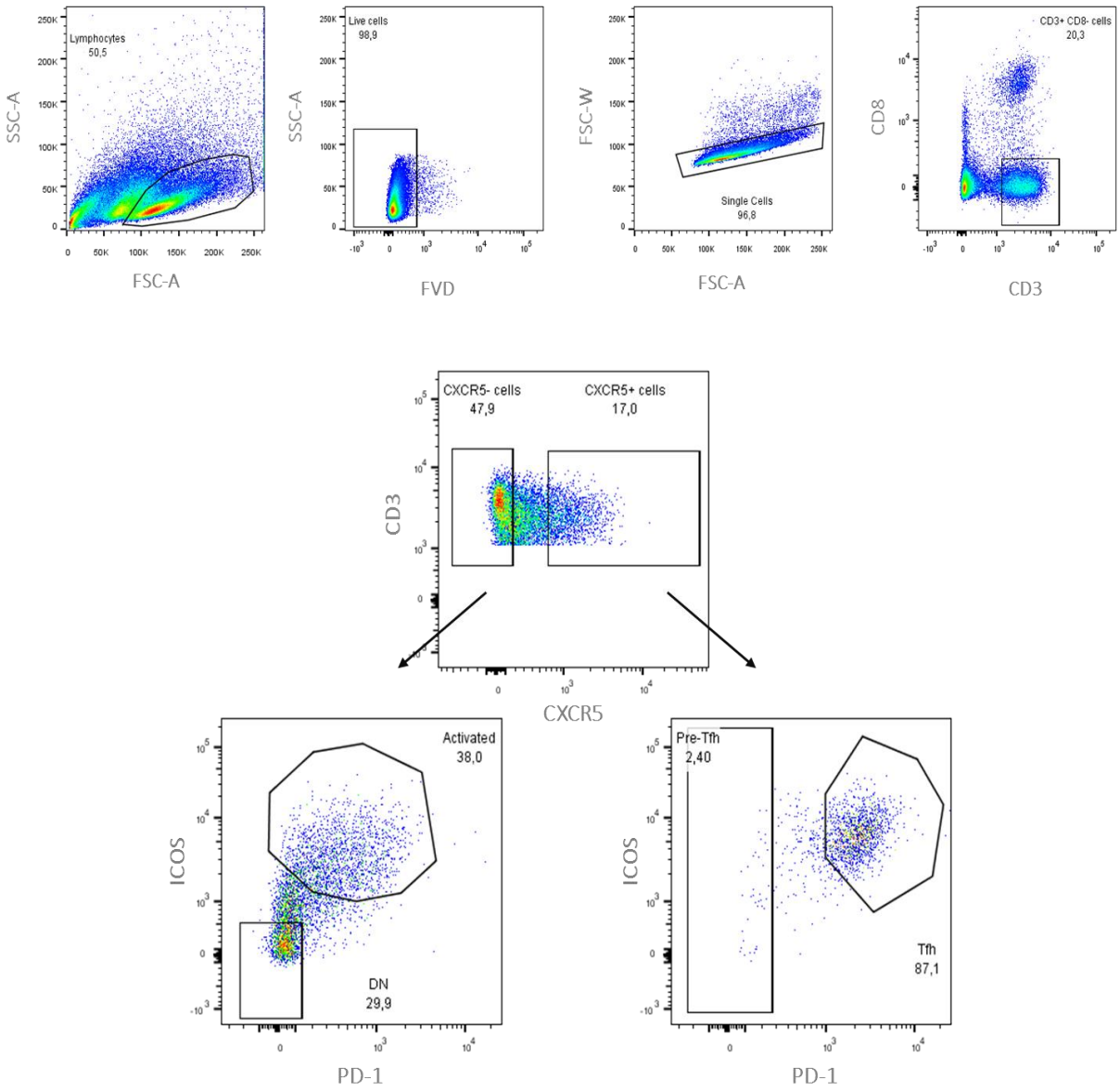

FIGURE 15 - FINAL SORTING STRATEGY. Cells subsets were obtained considering CXCR5, PD-1 and ICOS expression, in a FACS Aria IIu cell sorter (BD Biosciences).

In addition, we confirmed that these new populations greatly overlapped with the gates of the previous sorting strategy (Figure 16).

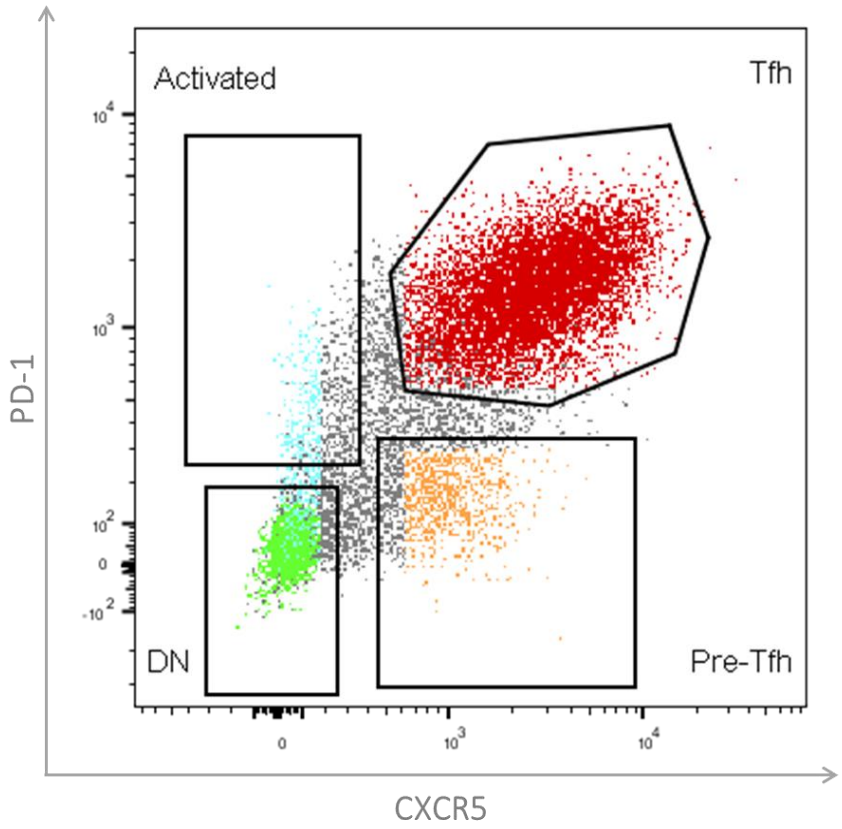

FIGURE 16 - POPULATIONS SORTED ACCORDING TO NEW SETTINGS OVERLAP WITH PREVIOUS POPULATIONS. The four cell subsets defined considering CXCR5, PD-1 and ICOS expression overlapped with the previous gate strategy and there was a correct correspondence between populations. Cells were acquired in a LSRFortessa cell analyzer (BD Biosciences) and data was analyzed by FlowJo Software (TreeStar).

In the interest of recovering a greater number of cells, the enrichment of CD4 cells was tested, using an untouched CD4 T cell isolation kit. The use of a negative selection kit allows infection of CD4+ T cell subsets since the CD4 receptor would be free to be engaged by HIV. In fact, this is the reason why CD8 antibody was used to sort CD4 T cell subsets. Both CD3+ CD8- and CD3+ CD4+ cell populations were found to be quite similar to each other which validates our gating strategy using CD8 as a marker (Figure 17).

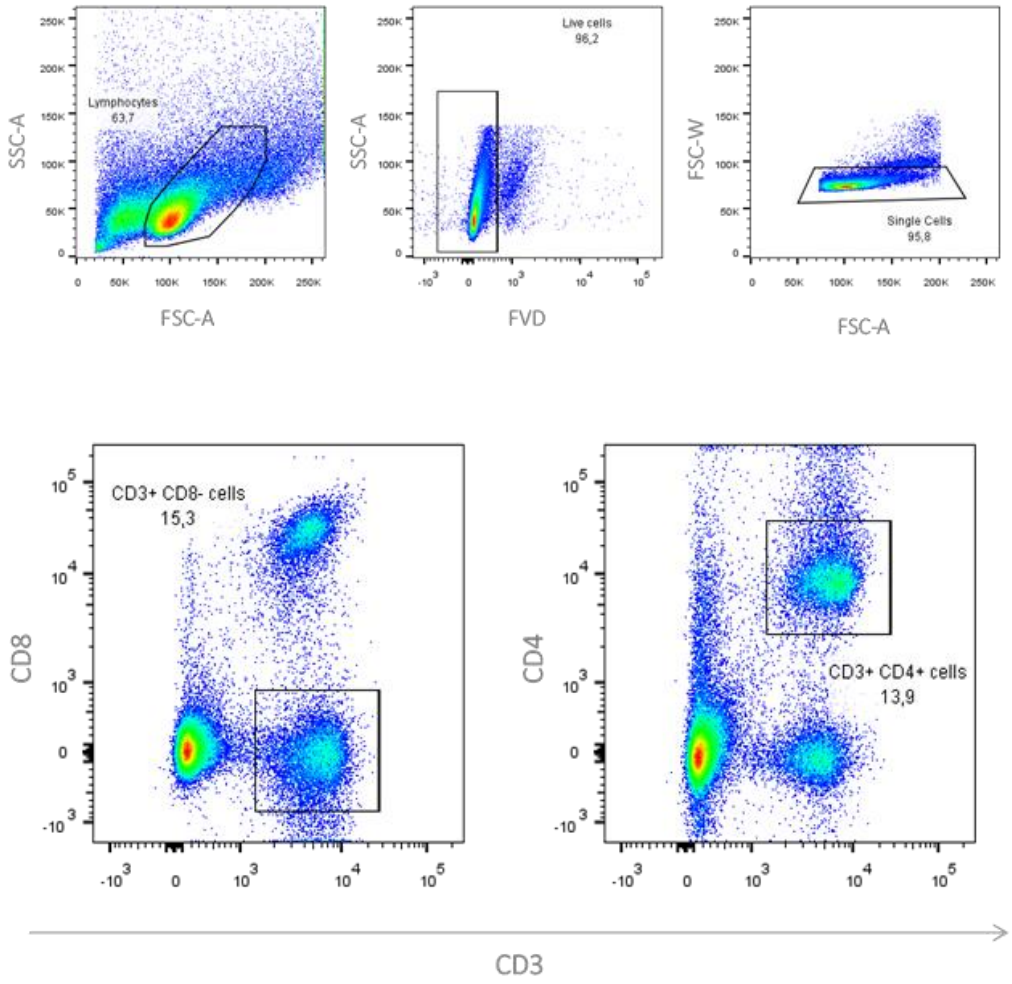

FIGURE 17 - PERCENTAGE OF CD3+ CD8- CELLS IS SIMILAR TO CD3+ CD4+ CELLS. TMNCs were stained against CD8 and CD4 molecules at the same time, to compare the percentages of both populations. Cells were aqcuired in a FACS Aria IIu cell sorter (BD Biosciences) and data was analyzed by FlowJo Software (TreeStar). Gate strategy is represented.

As shown in Figure 18, the enrichment increased the recovery of CD4+ T cell subsets as the CD3+ CD8- cell population is 6-fold higher after enrichment as compared to pre-enriched TMNCs. Of note, the enrichment process did not change the profile of the desired subsets, validating this approach. Moreover, the gating strategy after enrichment allowed us to recover the four CD4+ T cell subsets, with purity values always above 90% (Figure 19).

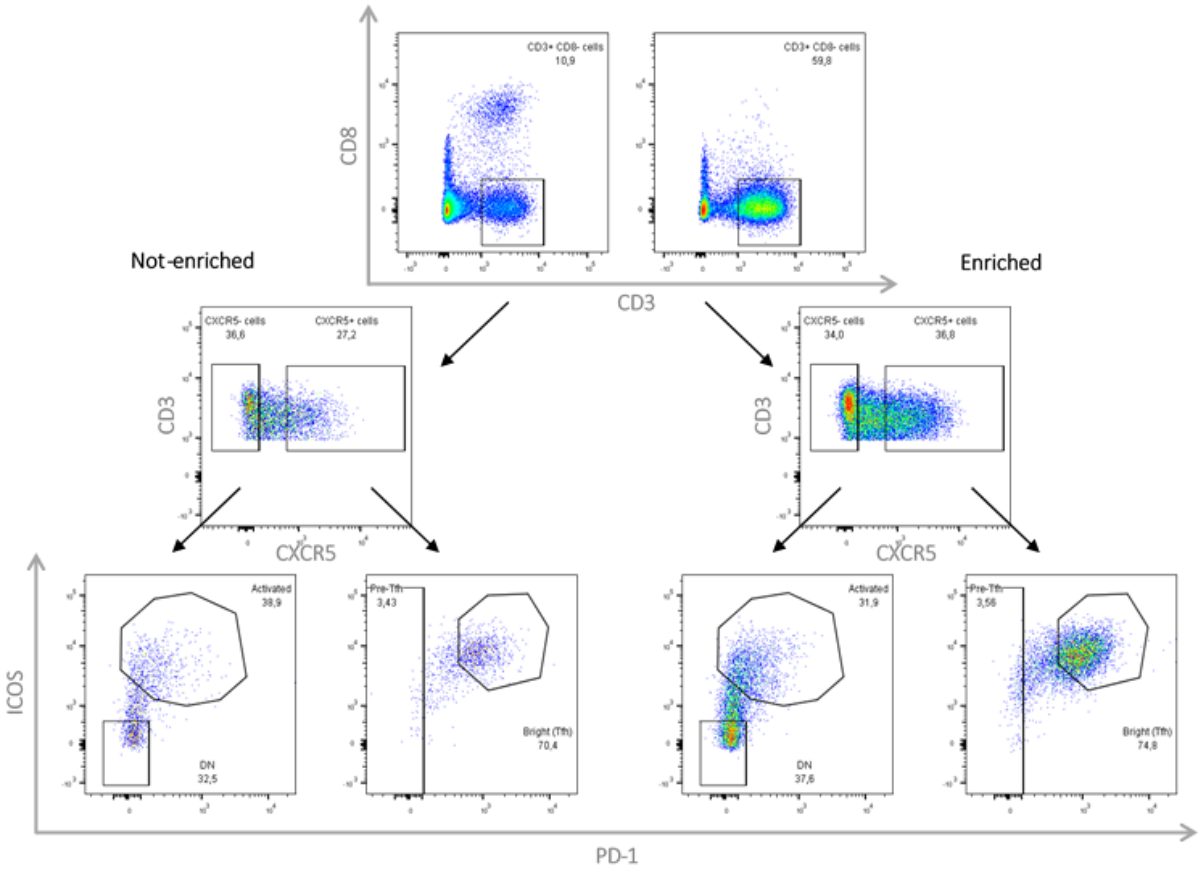

FIGURE 19 - ENRICHMENT INCREASES CD3+ CD8- CELL POPULATION BY APPROXIMATELY 6-FOLD. TMNCs were thawed on the day before of the enrichment. The Human CD4 T Cell Isolation Kit – MojoSort™ Magnetic Cell Separation System (Cat No. 480010, BioLegend) was used according to manufacturers' instructions. Cells were acquired in a FACS Aria IIu cell sorter (BD Biosciences) and data was analyzed by FlowJo Software (TreeStar). Representative data of one out of five experiments.

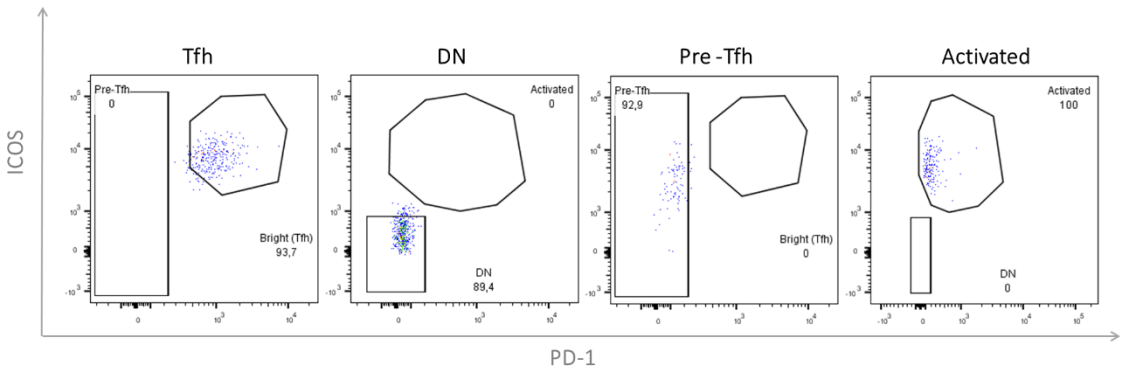

FIGURE 19 - CELL POPULATIONS PRESENT OVER 90% OF PURITY. Purity of cell subsets was analyzed in all experiments. Cells were acquired in a FACS Aria IIu cell sorter (BD Biosciences). Representative data of one out of five experiments.

4.2. EXPERIMENTAL DESIGN

After optimization of all steps to carry out the study (viral stock production, assessment of infection conditions, assessment of cell culture conditions, characterization of populations and definition of the sorting strategy), a final protocol was designed (Figure 21). In summary, we generated a biobank of frozen TMNCs immediately after tonsil collection and processing. On the day before the sorting, TMNCs were thawed and cultured overnight in CM_{cells} at a density of 10 x 10^6 cells/mL. On the next day, the CD4 T cell population was enriched for sorting of the four CD4 T subsets before infection. It is important to note that the infection experiments were decided according to the number of cells obtained after the sorting, in order to infect the largest number of populations and to use as many different viruses as possible. Table 5 summarizes the populations and viruses used in each experiment.

TABLE 5 – COMPILATION OF THE INFECTION EXPERIMENTS PERFORMED.

Virus			Experiment 1				Experiment 2				Experiment 3			
			Tfh	**DN**	**Pre-Tfh**	**Activated**	**Tfh**	**DN**	**Pre-Tfh**	**Activate**	**Tfh**	**DN**	**Pre-Tfh**	**Activated**
HIV-2	CXCR4	20.04	X				X				X	X		
		HSM10	X	X			X	X	X	X	X	X	X	X
	CCR5	60415K	X				X				X	X		X
HIV-1	CXCR4	92HT599	X	X			X	X			X	X	X	X
	CCR5	92HT660	X				X				X	X		X

After 24 hours of infection, cells from each population were stimulated after collecting a minimum number of cells to store as dry pellets. After 48h of stimulation (i.e. 72h of infection), supernatants were collected for viral infectivity assessment. In addition, cells were also collected at this point. Cell pellets were used for DNA and RNA extraction and subsequent quantification of total viral DNA.

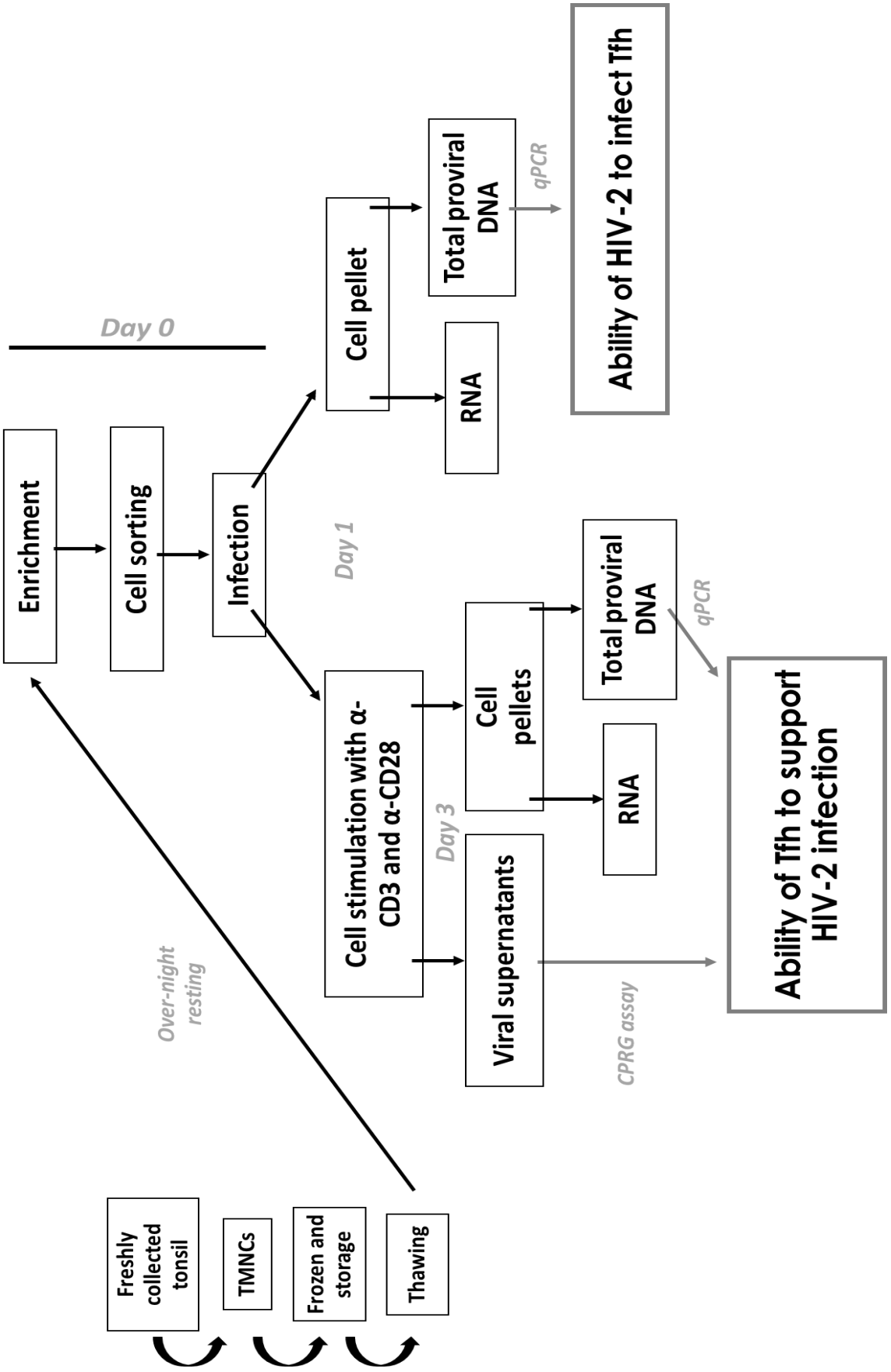

FIGURE 20 - SCHEMATIC DIAGRAM OF THE EXPERIMENTAL PROTOCOL.

4.3. ABILITY OF HIV-2 TO INFECT TFH CELLS

CD4 T cell subsets were infected with HIV-2 primary isolates with different coreceptors usage. Two X4-tropic viruses ($X4_{20.04}$ and $X4_{HSM10}$) and one R5-tropic virus ($R5_{60415K}$) were used. As observed in figure 21, all populations appeared to be permissive to all HIV-2 primary isolates even though the range of amounts of *gag* copies varies between primary isolates. Interestingly, Tfh cells were apparently more susceptible to HIV-2 infection compared to DN cells, independently of viral tropism.

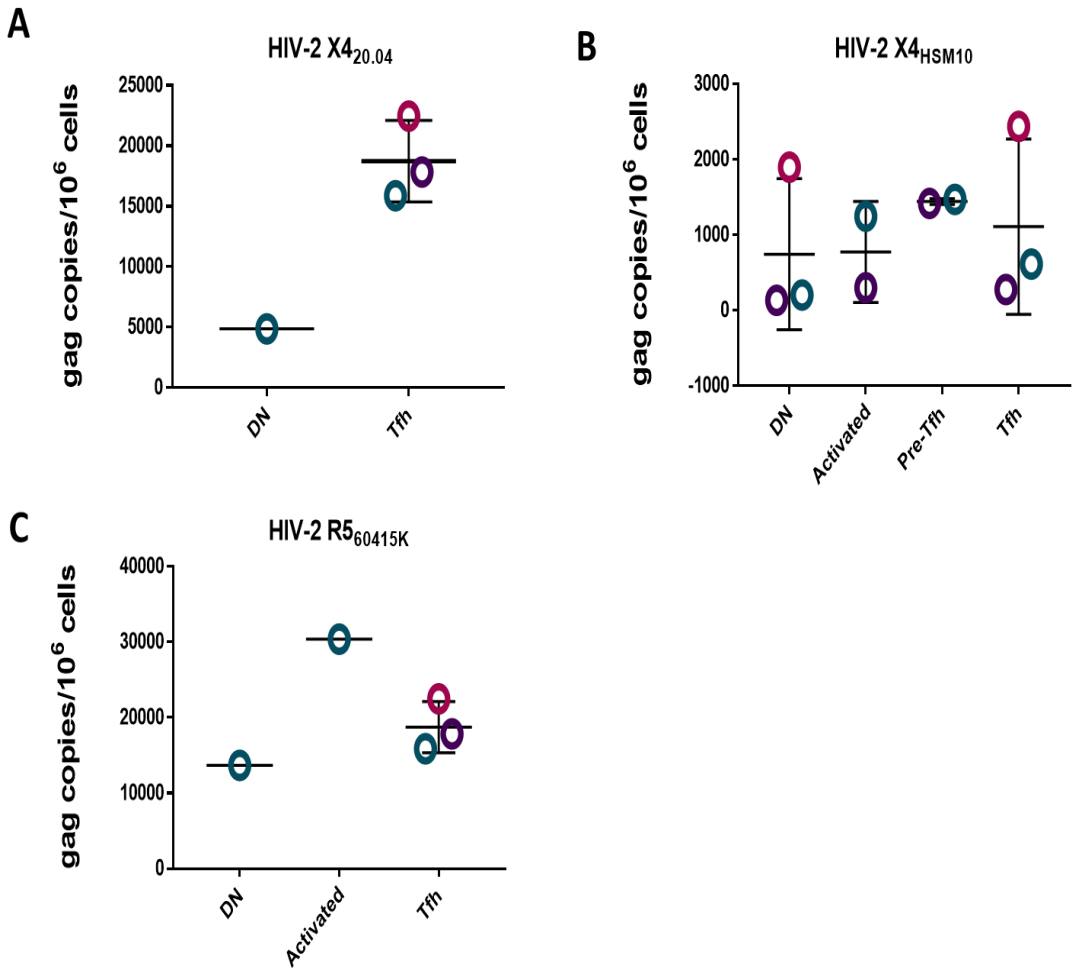

FIGURE 21 - HIV-2 IS ABLE TO INFECT TFH CELLS AS WELL OTHER CELL SUBSETS. Cells were infected with 1.6ng/10^6 cells of HIV-2 primary isolates X4-tropic 20.04 (A), X4-tropic HSM10 (B) or R5-tropic 60415k (C). After 24 hours of infection cellswere collected, DNA was extracted and total viral DNA levels were determined by TaqMan qPCR, using an ABI 7500 Real-Time PCR System (Applied Biosystems). Data were analyzed by GraphPad Prism. Pink: Experiment 1; Purple: Experiment 2; Blue: Experiment 3.

In parallel, cells were infected with HIV-1 primary isolates. Figure 22 shows that all CD4 T populations produced high amounts of *gag* copies, indicating that both X4- and R5 -tropic viruses were able to infect all tonsillar CD4 T subsets. Again, Tfh cells presented high number of *gag* copies although it was not clear if they were significantly more susceptible to infection than DN cells.

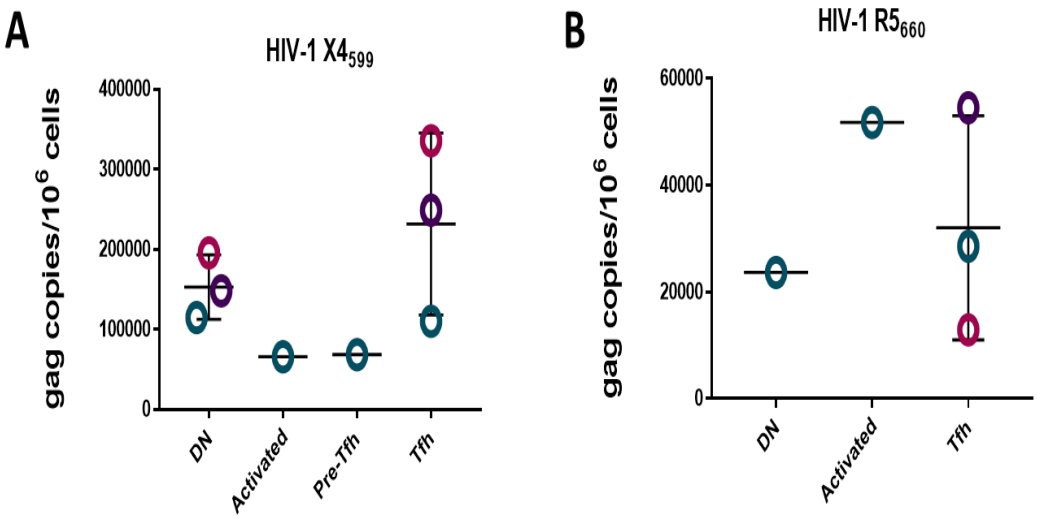

FIGURE 22 - HIV-1 INFECTION OF CD4 T CEL SUBSETS. Cells were infected with 1.6ng/10^6 cells of HIV-1 primary isolates X4-tropic 92HT599 (A) or R5-tropic 92US660 (B). After 24 hours of infection cell pellets were collected, DNA was extracted and total viral DNA levels were determined by qPCR, using an ABI 7500 Real-Time PCR System (Applied Biosystems). Data were analyzed by GraphPad Prism. Pink: Experiment 1; Purple: Experiment 2; Blue: Experiment 3.

When comparing X4-tropic viruses, Tfh cells appeared to be more susceptible to HIV-1 than HIV-2 infection (Figure 23) as total HIV DNA detected after HIV-1 $X4_{599}$ infection is 10 to 50-fold higher than in HIV-2 $X4_{20.04}$ and $X4_{HSM10}$ isolates. As expected from previous data in our lab, HIV-1 X4-tropic virus presented higher number of *gag* copies than HIV-1 R5-tropic virus, which is consistent to a higher expression of CXCR4 in Tfh cells as compared to CCR5 expression. Surprisingly, Tfh cells seemed to be more permissive to HIV-2 infection by R5-tropic virus (Figure 23.A). DN cells followed the same tendency than Tfh cells (Figure 23.B), although levels of total viral DNA suggested to be lower when compared to infected Tfh cells.

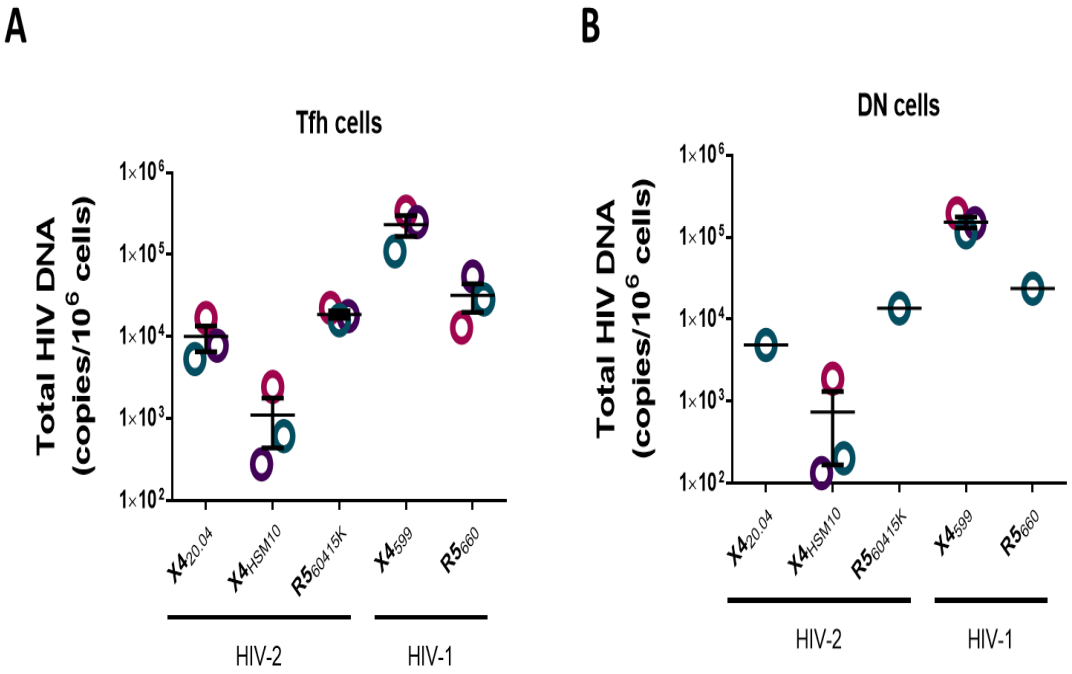

FIGURE 23 - HIV-1- INFECTED TFH AND DN CELLS PRESENT HIGHER LEVELS OF TOTAL HIV DNA THAN HIV-2-INFECTED CELLS. Tfh cells (A) and DN cells (B) were infected with 1.6ng/10^6 cells of each HIV primary isolates. After 24 hours of infection cell pellets were collected, DNA was extracted and total viral DNA levels were determined by qPCR, using an ABI 7500 Real-Time PCR System (Applied Biosystems). Data were analyzed by GraphPad Prism. Pink: Experiment 1; Purple: Experiment 2; Blue: Experiment 3.

4.4. ABILITY OF TFH CELLS TO SUPPORT PRODUCTIVE HIV-2 INFECTION

In order to determine whether Tfh cells were able to support a productive HIV-2 infection, infected cells were stimulated with α-CD3 and α-CD28 after 24 hours of infection, and maintained in culture for two days. Independently of the viral tropism, the Tfh subset appeared to support HIV-2 infection (Figure 24.A). The HIV-1 control also showed that Tfh were able to support HIV-1 infection (Figure 24.C).

In terms of DN cells, this subset was less able to support $X4_{20.04}$ and $R5_{60415K}$ productive infections, whereas $X4_{HSM10}$ infection results were inconclusive (Figure 24.B). Additionally, HIV-1 infection also appeared not to be supported by these cells (Figure 24.D).

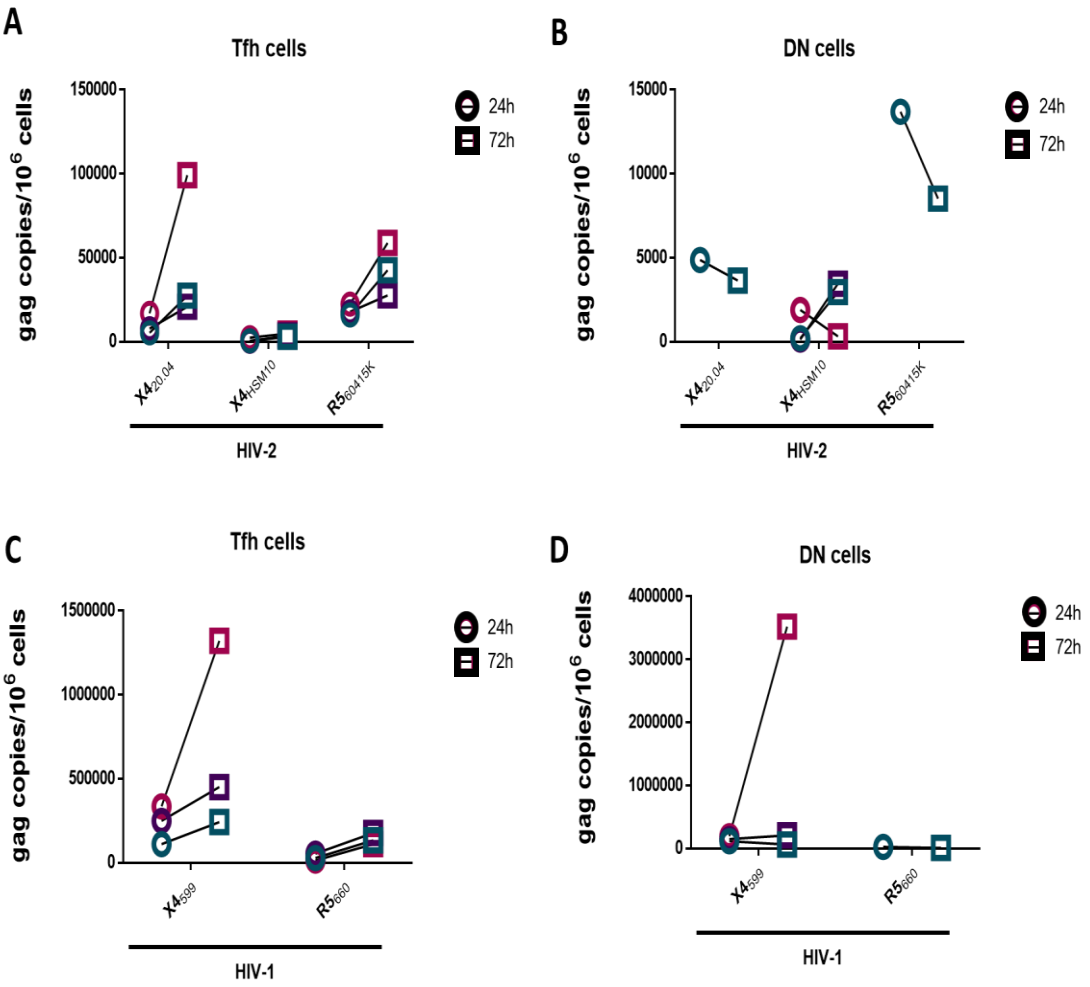

FIGURE 24 - CELLS' ABILITY TO SUPPORT PRODUCTIVE HIV INFECTION. After 24 hours of infection, cells were stimulated with 1µg/mL of α-CD3 (eBioscience) and cultured with 1µg/mL of α-CD28 (eBioscience) for two days. Then, cellswere collected, DNA was extracted and total viral DNA levels were determined by qPCR, using an ABI 7500 Real-Time PCR System (Applied Biosystems). Comparison of total viral DNA at 24h and at 72h post-infection by HIV-2 primary isolates on Tfh cells (A) or DN cells (B) or by HIV-1 primary isolates on Tfh cells (C) or DN cells (D). Data were analyzed by GraphPad Prism. Pink: Experiment 1; Purple: Experiment 2; Blue: Experiment 3.

We also assessed the infectivity of the newly virus particles produced. For this, we used the supernatants of each infection that we had collected and stored at -80°C to infect the TZM-bl reporter cell line. After 40h of infection, β-*galactosidase* expressed upon LTR transactivation from new virions was measured by Chlorophenol red-β-D-galactopyranoside (CPRG) colorimetric assay. Figure 25 shows that all new viruses produced from the different cell populations were infectious.

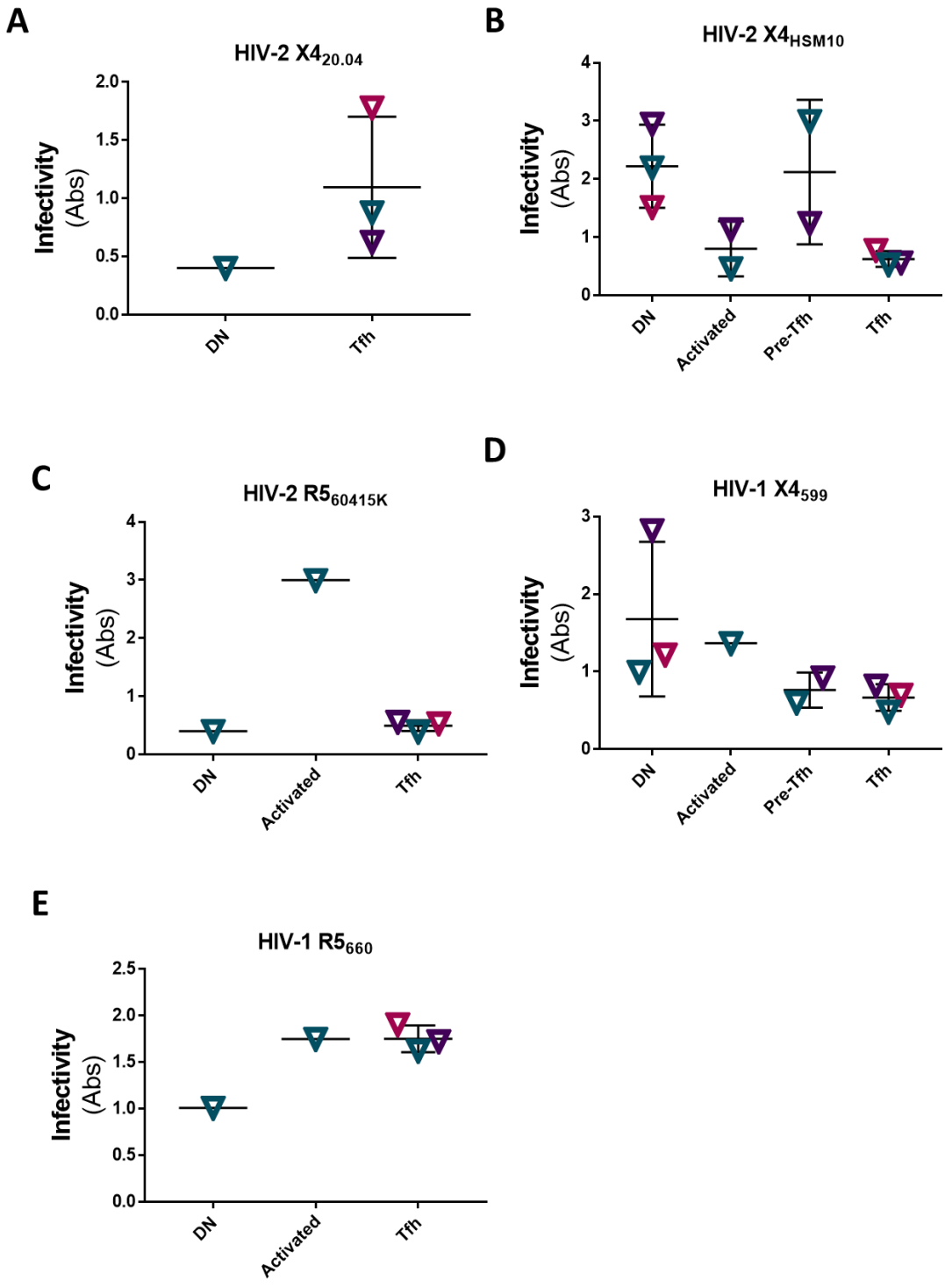

FIGURE 25 - NEWLY VIRAL PARTICLES ARE INFECTIOUS. TZM-bl reporter cell line was infected with the supernatants of each infection with HIV-2 primary isolates X4-tropic 20.04 (A), X4-tropic HSM10 (B) or R5-tropic 60415k (C) and HIV-1 primary isolates X4-tropic 92HT599 (D) or R5-tropic 92US660 (E). After 40h of infection, β-*galactosidase* expressed upon LTR transactivation from new virions was measured by Chlorophenol red-β-D-galactopyranoside (CPRG) colorimetric assay, using the Infinite M200 plate reader (Tecan). Data were analized by GraphPad Prism. Pink: Experiment 1; Purple: Experiment 2; Blue: Experiment 3.

We further evaluated whether there was a correlation between amounts of total viral DNA and virion production among different viruses and different subsets. However, there was no correlation between total viral DNA levels and the values of absorbance in HIV-2 infection or HIV-1 infections (r= - 0,172, P=0,4814; r= - 0,5245, P= 0,0839, respectively). Even comparing only Tfh cells it was not observed any correlation either in HIV-2 (r= 0,1333, P= 0,7435) or HIV-1 (r= - 0,7714, P= 0,1028) infections.

5. DISCUSSION AND FUTURE PERSPECTIVES

This work led to the design and optimization of a complete protocol that allows the study of purified CD4 + T cell subsets in the context of HIV infection.

The purpose of the study was to evaluate the ability of HIV-2 virus to infect Tfh cells. Some of the experimental options deserve further discussion.

Starting with viral stock production, co-cultures of PBMCs from three different individuals were used. Expression of some molecules that are determinant for infection, namely the expression of coreceptors (CXCR4 and CCR5) varies from individual to individual. Thus, by using cells from different donors, besides increasing the variability of the samples (which would be advantageous in this case), the cellular activation was also increased, which facilitates the infection.

The assay chosen to quantify the viral stock also has a rationale. When infecting the populations of interest, the aim was to infect each population with the same ammount of viral particles. The SG-PERT technique is equally sensitive to HIV-1 and HIV-2 viruses. Furthermore, it measures the RT activity, which provides an indirect hint if the virus is functional. Other authors have quantified viral stocks by the p24 antigen capture assay[84]. Although this assay is one of the most used to demonstrate replication of HIV-1[106], its sensitivity for detecting HIV-2 is not as effective since this virus produces the p27 protein, and the sensitivity of an ELISA based on p27 was shown to be lower than the one based on p24[82]. Moreover, detection of monomers from these proteins that are possibly being produced results in an erroneous virus quantification.

In other works, cell infection was performed by spinoculation, to increase the efficiency of the infection through the concentration of virus[87]. Nevertheless, this method may be at the origin of cellular and virus alterations[107]. In this work, both TMNCs and cells subsets were stimulated with polybrene, which was also found to increase the efficiency of infection[108], without affecting cells. However, if our study focused on issues related to virus entry, it would have to avoided.

During the protocol optimization, populations were characterized for the expression of several molecules, which also contributed to take some decisions regarding the sorting strategy.

According to what is already described, we have shown that Tfh express Bcl-6 while the other subsets (DN, Pre-Tfh and "Activated" cells) only show residual levels of this transcription factor[87,92].

Expression of CXCR4 and CCR5 coreceptors in Tfh and DN subsets was also assessed. We have demonstrated that Tfh express high levels of CXCR4, whereas DN cells present lower levels of this molecule, according to previous studies[87]. Regarding the expression of CCR5, both populations exhibited similar levels of this coreceptor, which is also in line with the study of Kohler, S. *et al* (2016).

Expression of CD57 was analyzed in the same populations and we have found that a subset within Tfh population expressed this marker. Since it is a marker characteristic of GC, it suggests that these cells are the Tfh cells which are present in the structure[97,104].

Expression of CD45RO was assessed and we found high levels on Tfh cells, as expected. In fact, some authors include this memory marker in the gate strategy to identify this population[94,109].

The same was observed for ICOS expression, which was evaluated not only in Tfh, but also in DN, Pre-Tfh and "Activated" cells. As reported by Perreau, M. *et al* (2013), the entire Tfh cell subset was found to express ICOS, as well as a large percentage of “Activated” cells. Unexpectedly, we found DN cells expressing CD45RO and ICOS, even if at low percentages. For this reason, we decided to include ICOS to better define the populations to be sorted, in order to obtain a negative cell population for the expression of the major Tfh markers.

Our results showed for the first time that HIV-2 is able to infect Tfh isolated from human tonsils. The comparison of the ability of HIV-2 and HIV-1 to infect Tfh suggests that the amount of proviral DNA generated is higher in the latter.

Our data on HIV-1 infection are in agreement with previous publications revealing high permissiveness of Tfh to HIV-1[92,95,110]. Besides, these cells are considered natural reservoirs of HIV-1[87,88,92,111].

When infected with the $X4_{20.04}$-tropic HIV-2 virus, Tfh appear to be the population most prone to infection as compared to other CD4+ T cell subsets in the tonsil. In the case of the $X4_{HSM10}$ virus, total proviral DNA levels do not appear to be far

apart among the four subsets. These findings require further investigation using other primary isolates. Moreover, it would be important to confirm that there are no imbalances in the efficiency of the PCR since the primers used in the amplification were originally designed specifically for the viruses that have been previously produced in the lab[70].

Concerning R5-tropic HIV-2, Tfh appear to be slightly more susceptible to infection than DN cells. Interestingly, the activated cells were the population that exhibited higher levels of total proviral DNA. As mentioned before, HIV mainly infects activated cells[79].

Unexpectedly, contrary to what we have seen in the case of HIV-1 infection, R5-tropic HIV-2 virus appears to be more infectious than R5-tropic HIV-1 when compared to the X4-tropic virus. This suggests that the biology of HIV-2 infection differs from HIV-1. Deeper studies regarding this topic would be relevant.

We also attempted to correlate the total proviral DNA values with the results of the infectivity assay of the newly produced viral particles (CPRG). However, no correlation was observed, although the results of CPRG support that the viruses were infectious. This could be an important read-out. In the case of HIV-2, Nunes-Cabaço, H. *et al* (2015), have shown that HIV-2 is able to infect the human thymus, but the HIV-2 replication cycle in thymocytes is impaired with a post-transcriptional block resulting in low viral load in culture supernatants. It would be important to further expand our results to evacuate this possibility.

One of the problems of this work was related to the heterogeneity of the tonsils. Although a prior validation of each sample had been performed, the behavior of each one in culture, for instance, can hugely differ, contributing to the variability of the results. More experiments will be needed to standardize the results.

This work also allowed the generation of material that will allow the realization of future studies. In parallel with the DNA extraction to quantify the proviral DNA, RNA samples were obtained that can be used to quantify HIV transcripts and to investigate functional aspects of Tfh. Changes related to the transcription of genes essential for cell function (i.e., Bcl-6, IL-21, etc.), as well as host restriction factors, which are part of the intrinsic defense mechanisms of the cell, can be studied.

It is also known that most of HIV-2-infected individuals are able to produce neutralizing Abs, contrary to what happens during HIV-1 infection[112]. Thus, co-culture studies of B-cells with infected and non-infected Tfh could be very useful to investigate possible differences in the impairment of Tfh ability to help B cells upon HIV-2 and HIV-1 infections.

The continuation of this line of research is crucial as there may be important discoveries involved in the discovery of new HIV vaccine strategies.

In conclusion, these findings contribute to a better understanding of HIV-2 infection, giving us new insights into the ability of the virus to infect Tfh cells and having identified new targets for viral replication control.

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