HIV persistence in mucosal T cells within the lungs of adults under long-term suppressive antiretroviral therapy

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ABSTRACT

Background: Cellular and anatomical reservoirs of HIV remain the primary challenge towards viral eradication. The lungs are important but relatively understudied anatomical reservoirs in the era of antiretroviral therapy (ART). Effector memory, memory $CCR6^+$ and $CD32a^+$ CD4 T cells, as well as double negative $CD4^-CD8^-$ T cells have been described as potential cellular reservoirs of HIV in the peripheral blood. We hypothesized that a higher HIV reservoir burden within the lungs *vs* the peripheral blood during long-term ART is due to a disrupted T cell immunity. Thus, we assessed the frequencies, distributions and phenotypic characteristics of pulmonary mucosal cellular subsets in both HIV-infected adults under long-term suppressive ART as well as healthy individuals.

Methods: Bronchoalveolar lavage (BAL) fluid and bronchial mucosal biopsies, obtained by bronchoscopy, and matched peripheral blood samples were collected from 24 HIV^+ individuals without respiratory symptoms and under long-term suppressive ART (undetectable plasma viral load and CD4 count >350 cells/mm^3 for ≥3 years) and from 8 HIV^- individuals without respiratory symptoms. T cell subsets were characterized by flow cytometry, and HIV DNA and RNA were assessed by PCR.

Results: A greater frequency of HIV DNA was observed in BAL cells compared to peripheral blood mononuclear cells and biopsies in ART-treated individuals. Importantly, HIV RNA was detected in BAL cells, and highly pure sorted BAL CD4 T cells harbored higher levels of HIV DNA than peripheral CD4 T cells and alveolar macrophages. Both study groups experienced a decrease in naïve CD4 T cells and an increase in effector memory CD4 T cells, the most well-recognized cellular reservoir of HIV, in the lungs compared to blood. Pulmonary mucosal CD4 T cells were found to be more activated and senescent, with an enrichment in $CD39^+$ regulatory T cells that inhibit anti-HIV specific responses. CD4 T cells expressing the Fc receptor CD32a, a recently proposed marker of CD4 T cells harboring replication-competent virus, were highly enriched in the pulmonary compartment of ART-treated adults *vs* blood, with greater activation levels and permissiveness to HIV. Furthermore, higher frequencies of memory $CCR6^+$ CD4 T cell subsets

described as HIV reservoirs were found within the lungs *vs* blood of both HIV^+ and HIV^- individuals. Moreover, a substantial increase in both $CD4^-CD8\alpha^-CD8\beta^-$ and $CD4^-CD8^-TCR\alpha\beta^-TCR\gamma\delta^-$ double negative T cells was observed in the lungs *vs* blood of HIV^+ individuals. These double negative T cells were characterized by higher levels of immune activation, lower levels of immune senescence and lower expression of the marker of recent thymus emigrants. Finally, a decrease in naïve CD8 T cells and an increase in effector memory CD8 T cells were observed among BAL cells compared to their circulating counterparts in both study groups. CD8 T cells were more activated and the CD73-expressing HIV-specific subset was found in lower frequency in the lungs compared to blood. Interestingly, in ART-treated participants, CD8 T cells were more exhausted and expressed less perforin and granzyme B.

Conclusion: In virally suppressed HIV^+ adults, the lungs contain higher levels of HIV DNA, specifically in CD4 T cells, and higher frequencies of various T cell subsets known as preferential HIV reservoirs, including effector memory, memory $CCR6^+$ and $CD32a^+$ CD4 T cells as well as activated double negative T cells when compared to peripheral blood. Furthermore, pulmonary CD8 T cells were found to be more exhausted and dysfunctional despite greater effector phenotype compared to peripheral CD8 T cells. In healthy adults, certain CD4 T cell HIV reservoirs exist in higher proportion compared to blood, while anti-HIV specific CD8 T cells were reduced in the lungs. The particular distribution and immune properties of mucosal T cells could contribute to the preferential persistence of HIV reservoirs in the lungs despite ART. The characterization of HIV reservoirs will allow us to develop tissue-targeted therapeutic strategies that may enable viral eradication in people living with HIV.

RÉSUMÉ

Contexte : Les réservoirs cellulaires et anatomiques de VIH demeurent le défi majeur vers l'éradication virale. Les poumons sont des réservoirs anatomiques importants mais relativement peu étudiés dans l'ère du traitement par antirétroviraux (ARV). Les cellules T CD4 effectrices mémoires, mémoires CCR6^{+} et CD32a^{+}, ainsi que les cellules T CD4^{-}CD8^{-} double négatives ont été décrites comme étant des réservoirs potentiels de VIH dans le sang périphérique. Nous avons émis l'hypothèse qu'un fardeau plus élevé du réservoir de VIH dans les poumons *vs* le sang périphérique durant le traitement par ARV à long-terme est due à une immunité perturbée des cellules T. Par conséquent, nous avons évalué les fréquences, distributions et caractéristiques phénotypiques des sous-ensembles de cellules muqueuses pulmonaires chez des adultes infectés par le VIH et sous traitement suppressif par ARV à long-terme ainsi que chez des individus sains.

Méthodes : Du fluide de lavage bronchoalvéolaire (LBA) et des biopsies muqueuses pulmonaires, obtenus par bronchoscopie, et des échantillons de sang périphérique appariés ont été recueillis chez 24 individus VIH^{+} sans symptômes respiratoires et sous traitement suppressif par ARV à long-terme (charge virale plasmatique indétectable et nombre de CD4 >350 cellules/mm^{3} pour ≥3 ans) et chez 8 individus VIH^{-} sans symptômes respiratoires. Les sous-ensembles de cellules T ont été caractérisés par cytométrie en flux, et l'ADN and l'ARN viraux ont été quantifiés par PCR.

Résultats : Une plus grande fréquence d'ADN de VIH a été observée dans les cellules du LBA comparées aux cellules mononuclées du sang périphérique et aux biopsies. Surtout, l'ARN virale a été détectée dans les cellules du LBA, et les cellules triées T CD4 du LBA abritaient des niveaux plus élevés d'ADN de VIH que les cellules T CD4 périphériques et les macrophages alvéolaires. Les deux groupes d'étude ont subi une réduction de cellules T CD4 naïves et une augmentation de cellules T CD4 effectrices mémoires, le réservoir cellulaire de VIH le mieux reconnu, dans les poumons comparés au sang. Les cellules T CD4 muqueuses pulmonaires sont plus activées et sénescentes, avec un enrichissement des cellules T régulatrices CD39^{+} qui inhibent les réponses spécifiques anti-VIH. Les cellules T CD4 exprimant le récepteur Fc CD32a, un marqueur proposé récemment de cellules T CD4 abritant du virus compétent pour la réplication, étaient hautement

enrichies dans le compartiment pulmonaire chez les adultes traités aux ARV *vs* le sang, avec une plus grande activation et permissivité au VIH. De plus, une fréquence plus élevée de sous-ensembles de cellules mémoires $CCR6^+$ T CD4 décrites comme réservoirs cellulaires de VIH a été trouvée dans les poumons *vs* le sang chez les individus VIH^+ et VIH^-. Par ailleurs, une hausse substantielle de cellules T double négatives $CD4^-CD8\alpha^-CD8\beta^-$ et $CD4^-CD8^-TCR\alpha\beta^-TCR\gamma\delta^-$ a été observée dans les poumons *vs* le sang chez les individus VIH^+. Ces cellules T double négatives sont caractérisées par des niveaux plus élevés d'activation immunitaire, des niveaux moins élevés de sénescence immunitaire et une expression inférieure du marqueur d'émigrants récents du thymus. Finalement, une réduction de cellules T CD8 naïves et une augmentation de cellules T CD8 effectrices mémoires ont été observées parmi les cellules du LBA comparées à leurs homologues en circulation chez les deux groupes d'étude. Les cellules T CD8 étaient plus activées et le sous-ensemble VIH-spécifique exprimant CD73 a été trouvé en fréquence moins élevé dans les poumons comparés au sang. Intéressement, chez les participants traités aux ARV, les cellules T CD8 sont plus épuisées et exprimaient moins de perforine et de granzyme B.

Conclusion : Chez les adultes VIH^+ avec une charge virale supprimée, les poumons contiennent des niveaux plus élevés d'ADN de VIH, spécifiquement dans les cellules T CD4, et des fréquences plus élevées de plusieurs sous-ensembles de cellules T connus comme réservoirs préférentiels de VIH, y compris les cellules T CD4 effectrices mémoires, mémoires $CCR6^+$ et $CD32a^+$ ainsi que les cellules activées T double négatives, en comparaison au sang périphérique. De plus, les cellules T CD8 pulmonaires étaient plus épuisées et dysfonctionnelles malgré un phénotype effecteur plus prononcé comparé au cellules T CD8 périphériques. Chez les adultes sains, certains réservoirs cellulaires T CD4 existent en plus grande proportions dans les poumons comparés au sang, tandis que les cellules T CD8 spécifiquement anti-VIH étaient diminuées dans les poumons. La distribution particulière et les propriétés immunitaires des cellules T muqueuses pourraient contribuer à la persistance préférentielle de réservoirs de VIH dans les poumons malgré le traitement par ARV. La caractérisation des réservoirs de VIH nous permettra de développer des stratégies thérapeutiques ciblant les tissues qui pourraient permettre l'éradication virale chez les personnes vivant avec le VIH.

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LIST OF ABBREVIATIONS

ADN (French): Acide désoxyribonucléique
AIDS: Acquired immunodeficiency syndrome
AMP: Adenosine monophosphate
ARN (French): Acide ribonucléique
ART: Antiretroviral therapy
ARV (French): Antirétroviral
ATP: Adenosine triphosphate
BAL: Bronchoalveolar lavage
CD: Cluster of differentiation
CHUM: Centre hospitalier de l'Université de Montréal
CM: Central memory
COPD: Chronic obstructive pulmonary disease
CROI: Conference on Retroviruses and Opportunistic Infections
DN: Double negative
DNA: Deoxyribonucleic acid
DP: Double positive
EM: Effector memory
ER: Endoplasmic reticulum
ERAD: Endoplasmic reticulum-associated degradation
HIV: Human immunodeficiency virus
IFN: Interferon
IL: Interleukin
FACS: Fluorescence-associated cell sorting
IQR: Interquartile range
LAG: Lymphocyte-activating gene
LBA (French): Lavage bronchoalvéolaire
MHC: Major histocompatibility complex
MUHC: McGill University Health Centre
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
PAMP: Pathogen-associated molecular pattern
PBMC: Peripheral blood mononuclear cells
PCR: Polymerase chain reaction
PD: Programmed death
RNA: Ribonucleic acid
RTE: Recent thymic emigrants
RT-PCR: Real-time polymerase chain reaction
SIV: Simian immunodeficiency syndrome
SHIV: Simian-human immunodeficiency syndrome
SP: Single positive
TB: Tuberculosis
TCR: T cell receptor
TD: Terminally differentiated
TGF: Transforming growth factor
Th: T helper
TNF: Tumor necrosis factor
Tregs: Regulatory T cells

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INTRODUCTION

Rationale

Reservoirs of HIV are the primary reason why HIV/AIDS remains an incurable disease today despite the success of antiretroviral therapy (ART). A reservoir may either be cellular or anatomical. Memory CD4 T cells are the best characterized cellular reservoirs as HIV can remain latent within the genome of the host cell in the form of proviral DNA (1). From an anatomical perspective, most attention has been dedicated to the central nervous system, the testicular tissue and the gut-associated lymphoid tissue. In contrast, despite being an immunological effector site, the lungs have received relatively little attention in the era of ART despite intense efforts aimed at understanding HIV reservoirs. The lungs exhibit key characteristics that make them ideal sites for HIV latency and persistence (2). Millions of alveoli are present in the lungs which create a very large surface area. Additionally, the close proximity of alveolar macrophages and pulmonary mucosal T cells, in which HIV is readily detectable in infected individuals, provide multiple occasions for cell-to-cell transfer of HIV. The genetic variants of HIV present in the lungs differ from those found in peripheral blood which suggests a certain degree of compartmentalization of the virus within the pulmonary site. Antiretroviral drugs penetrate the lungs with differential efficiency based on lipid solubility. Finally, *in vitro* studies demonstrated that co-infections and tobacco usage induces greater viral replication, which allows for the seeding of latent provirus and the development of viral escape mutations. HIV persistence is one of the main causes of chronic systemic inflammation and immune activation even following successful ART for over a decade. HIV-infected individuals display various pulmonary immune perturbations, including imbalances in the frequencies of various immune cells and their dysfunction, which contribute to greater production of various pro-inflammatory cytokines within the lungs (2). Furthermore, as HIV-infected individuals are living longer thanks to ART, there has been an emergence of many diseases associated with aging, including chronic obstructive pulmonary disease (3), the third leading cause of death in North America. It is therefore of paramount importance to gain a comprehensive understanding of the immune milieu within the lungs in HIV-infected individuals in relationship to the intrapulmonary HIV

reservoir burden, as the interplay between pulmonary inflammation, imbalances in immune cell frequencies and the size of the lung HIV reservoir of individuals on ART has never been explored previously.

Hypothesis

Higher HIV reservoir burden within the lungs *vs* the peripheral blood of HIV-infected adults under long-term suppressive ART is due to a disrupted T cell pulmonary mucosal immunity.

Main objectives

(1) To evaluate the levels of HIV persistence in pulmonary mucosal CD4 T cells compared to alveolar macrophages and peripheral CD4 T cells under long-term suppressive ART.

(2) To characterise pulmonary mucosal CD4, CD8 and double negative T cell subsets in HIV-infected adults receiving long-term suppressive ART and in healthy individuals.

PREFACE

This thesis conforms to the McGill University Graduate and Post-Doctoral Studies' guidelines for thesis preparation.

Author contribution for Chapter 1 Literature Review:
The chapter was written by Syim Salahuddin, with suggestions from Dr. Cecilia T. Costiniuk and Dr. Mohammad-Ali Jenabian.

Author contribution for Chapter 2 Methodology:
The methodologies presented were designed by Syim Salahuddin, Dr. Cecilia Costiniuk, Dr. Mohammad-Ali Jenabian and Dr. Nicolas Chomont.

Author contribution for Chapter 3 Research Findings:
The experiments were conducted by Syim Salahuddin, Dr. Omar Farnos and Amélie Pagliuzza. Analyses and interpretation of the data were performed by Syim Salahuddin, Dr. Cecilia Costiniuk, Dr. Mohammad-Ali Jenabian and Dr. Nicolas Chomont.

Author contribution for Chapter 4 Discussion:
The chapter was written by Syim Salahuddin, with suggestions from Dr. Cecilia T. Costiniuk and Dr. Mohammad-Ali Jenabian.

CHAPTER 1: LITERATURE REVIEW

Author contribution: The following chapter was written by Syim Salahuddin, with suggestions from Dr. Cecilia T. Costiniuk and Dr. Mohammad-Ali Jenabian.

1.1 HIV & AIDS

1.1.1 Discovery of HIV

The human immunodeficiency virus (HIV) is a retrovirus that progressively causes the acquired immunodeficiency syndrome (AIDS) in humans if left untreated. The virus first emerged in the early 20th century through zoonotic simian immunodeficiency virus (SIV) infection of humans living in Western and Central Africa (4). HIV-1 group M, which represents a specific transmission event and is the origin of the current worldwide HIV/AIDS pandemic, arose from chimpanzees infected with SIV (5). Following the cross-species transmission, HIV-1 underwent genetic diversification into subtypes, while remaining confined to the sites of origin within Africa (6). The worldwide spread of HIV-1 group M subsequently occurred in the second half of the 20th century, resulting in a genetically and recombinantly diverse geographic distribution of the pandemic group (7). The diversity of HIV-1 has significant clinical implications as the subtypes display differing transmission rates (8), disease progression (9), diagnostic implications and viral load set points (10), viral load suppression rates during antiretroviral therapy (ART) (11) and development of drug resistance mutations (12). The clinical features associated with the immunodeficient patterns of HIV/AIDS were first identified in 1981 (13) and HIV itself was subsequently isolated in 1983 (14) by the 2008 Nobel Prize in Physiology or Medicine laureates Françoise Barré-Sinoussi and Luc Montagnier in their lab at Institut Pasteur, Paris, France. Based on the patterns of immune compromise previously healthy individuals were displaying post-infection, it became clear that HIV induced a cellular abnormality in immune function (15).

1.1.2 Epidemiology of HIV

Since its identification, HIV has been one of the largest public health concerns in history, with over 76 million infections and 35 million deaths. By the end of 2016, 36.7 million people were living with HIV worldwide, with almost 2 million new infections and 1 million AIDS-related deaths that year (16). In 2015, HIV/AIDS was the fifth leading cause of death in low-income

economies (17), which along with middle-income nations contain the vast majority of individuals living with the virus in the world. The most affected region in the world is sub-Saharan Africa (Figure 1).

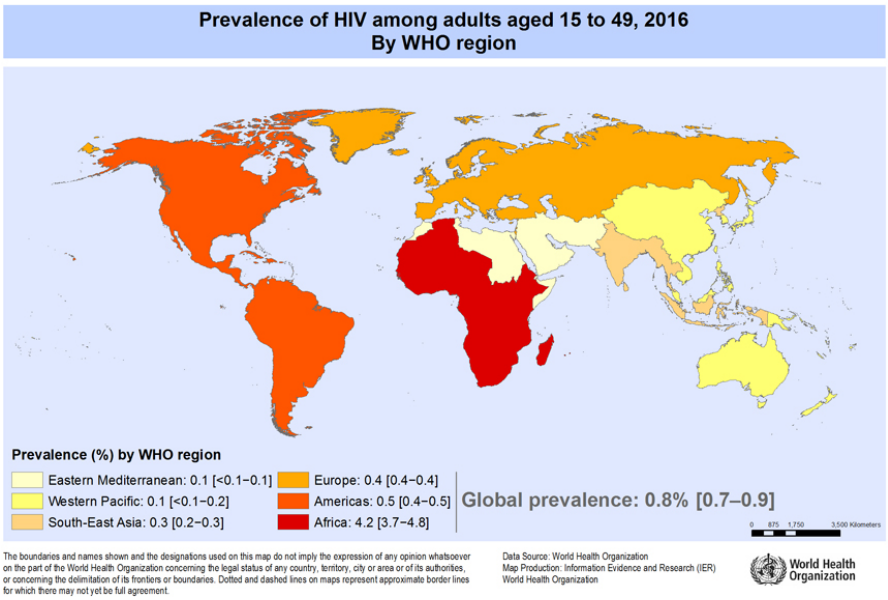

Figure 1: Regional and global prevalence of HIV among people aged 15 to 49 in 2016 (18).

In Canada, there were an estimated 65,040 HIV-infected persons by the end of 2014. Of that number, 20% of these individuals remained undiagnosed and 24% of those diagnosed were still not on ART (19) despite living in a high-income country. The primary attributable risk factor of HIV acquisition was being a man having sex with another man, followed by injection drug use and heterosexual sex, though this distribution varies from region to region (20). Although now rare in Canada, HIV may also be transmitted transplacentally from mother to child or through infected blood. Together, these epidemiological data corroborate the global effort already invested and still needed in HIV/AIDS research, education and prevention.

1.1.3 Life cycle of HIV-1

The HIV-1 virion carries its full-length genome in the form of a single-stranded RNA that contains genes encoding a total of 16 proteins needed in the replication cycle of the virus. These

proteins include structural proteins encoded by *gag*, enzymatic proteins encoded by *pol*, envelope proteins encoded by *env*, regulatory proteins encoded by *tat* and *rev*, and accessory proteins encoded by *vif, vpu, vpr* and *nef* (Figure 2) (21).

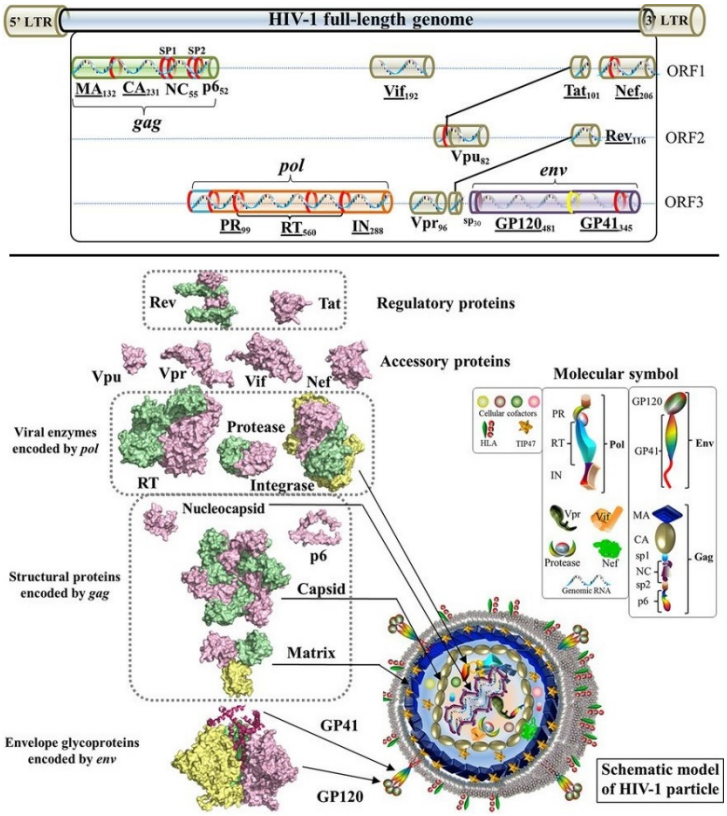

Figure 2: Full-length HIV-1 genome (top) and proteins encoded by the genes in the viral genome (bottom). Adapted from (21).

The replication cycle of HIV-1 can be dichotomized into two phases: the early phase, wherein the virus enters the target cell and integrates its DNA within the host genome inside the nucleus, and the late phase, which comprises the expression of integrated viral DNA and subsequent release of new viral entities by the infected cells. The early phase begins with HIV-1 attaching itself to a host cell through specific or nonspecific interactions mediated by either its envelope protein, Env, or the host cell's membrane proteins (22). Following adhesion, Env binds with the cluster of differentiation (CD) 4, a glycoprotein that constitutes its primary receptor, and with co-receptors that bolster Env to fuse with the host membrane. There are two such co-

receptors, the chemokine receptor CCR5 and the chemokine receptor CXCR4, which may be used alone or together depending on the phenotype of the infecting viral strain (23). Following localization of the virion to a site for optimal fusion, a process aided by the host cell's machinery, the virus fuses with the plasma membrane and enters the cytoplasm (22). Shortly after the content of the virion is spilt in the cytosol, reverse transcription occurs. Indeed, the HIV-1 reverse transcriptase, a product of the GagPol polyprotein, converts the single-stranded RNA found inside the virion into a double-stranded DNA substrate used later in integration (24). Once DNA synthesis is completed in the reverse transcription complex, the latter becomes a pre-integration complex which migrates into the nucleus (24). This nucleoprotein complex contains an integrase, another viral protein originating from GagPol and released by the virion along with reverse transcriptase. The enzyme associates with the newly synthesized viral DNA and can insert the molecule at various sites within the host genome (25). This process can be aided by co-factor proteins already found within the host cell (26).

The late phase of the HIV-1 life cycle begins with the transcription of the integrated provirus mediated by the virally encoded Tat protein, its interactions with cellular co-factors, and epigenetic modifications (27). The resulting messenger RNA transcripts are then exported out of the nucleus by another viral protein, Rev, which binds to a stem-loop structure within the RNA molecule and transports it through nuclear pore complexes (28). Viral RNAs transcribed in the nucleus enter the cytosol and can achieve two goals. Indeed, some HIV-1 messenger RNAs are translated into viral proteins that participate as mediators or substrates in virus assembly and release (29), while others dimerize to be packaged into maturing virions (30). The first goal sees the expression of the Gag precursor protein that forms the viral particle and, at lower levels, of the GagPol precursor polyprotein that forms the enzymes utilized in the next life cycle of the virus, namely in integration, transcription and protein cleavage (31). Following mutational changes in Gag, it was suggested the matrix domain within the precursor protein may play a crucial role in the targeting of Gag to the plasma membrane (32). Similarly, reduction in the expression of phosphatidylinositol-4,5-bisphosphate found at high levels on the intracellular side of the plasma membrane has also resulted in significantly depleted viral production, hinting at

the existence of cellular players in Gag recruitment (33). Upon localization to the plasma membrane, Gag interacts with specialized regions of the membrane called lipid rafts (34) that play a crucial role in HIV-1 replication (35), especially since virological synapses are notably enriched in these rafts (36). Thus, these lipid rafts may be used as sites for the formation of new virions. Two copies of viral RNA are packaged inside the virions along with protease, reverse transcriptase and integrase; these enzymes exist as domains of GagPol. Thus, after assembling itself with the two viral RNA copies and the GagPol precursor protein, Gag aligns itself radially to form a spherical lattice. The viral envelope protein Env, found at the lipid raft sites, joins the assemblage and the nascent viral particle is released by the host cell through membrane fission (37). Finally, proteolysis of Gag by the packaged protease stabilizes the viral RNA transcripts, forms a capsid structure around the packaged elements, and ultimately completes the maturation of the new HIV-1 virus which can now infect its next cellular target (38). The steps of the HIV-1 life cycle are summarized in Figure 3. In addition to CD4 T cells, which constitute their primary cellular targets, HIV-1 virions have the ability to infect other cell types such as macrophages and monocytes (39), dendritic cells (40) and astrocytes (41).

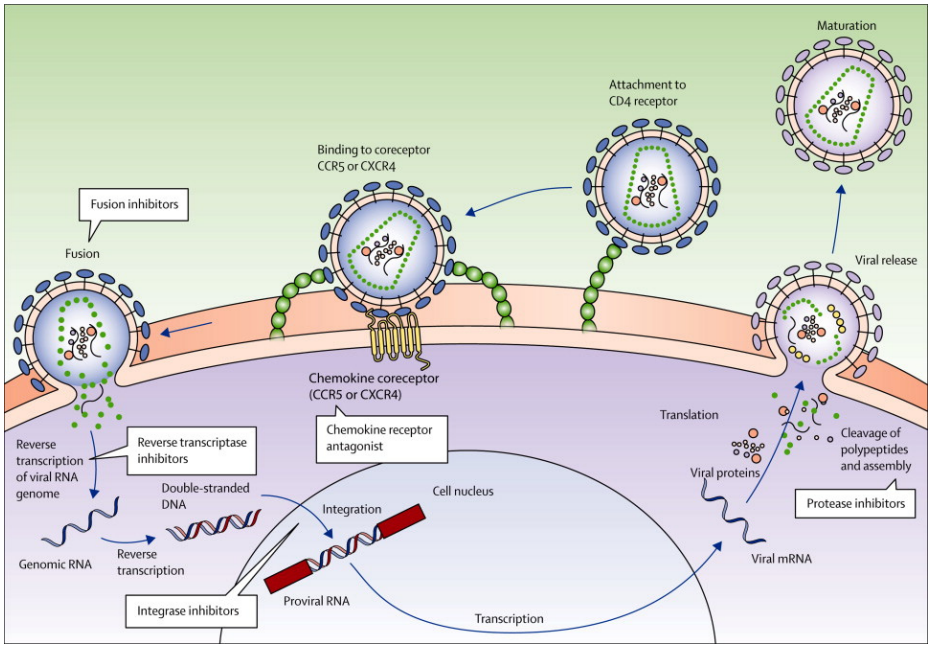

Figure 3: Steps in the early and late phase of the life cycle of HIV-1, with associated mechanisms of action of antiretroviral drugs (42).

1.1.4 Pathogenesis of HIV

Following infection, the virus quickly begins rounds of replication at the site of transmission. At the mucosal level for instance, the constant replenishment of memory CD4 T cells in the lamina propria allows the virus to replicate rapidly (43), subsequently propagating to draining lymph nodes and other lymphoid tissues. The gut-associated lymphoid tissue, which bears about 60% of the body's CD4 T cells, is especially affected by acute HIV infection as it experiences a dramatic loss of CD4 T cells (44) with only partial reconstitution during ART (45). The structural integrity of the gut mucosa is also affected, which allows for the translocation of microbial products from the gut to the peripheral circulation (46). Indeed, the levels of circulating lipopolysaccharide is increased and correlated with both innate and adaptive immune activation (46). HIV infection further perturbs the production of inflammatory cytokines and chemokines, resulting in the elevation of plasma levels of various proinflammatory factors (47). During acute HIV infection, people often experience fever, enlarged lymph nodes, sore throat, myalgias, arthralgias, headaches and diarrhea, all collectively known as a retroviral syndrome and usually occurring within 2 to 4 weeks (48).Once the innate and adaptive immune responses are initiated, the viral load decreases while CD8 T cells begin to kill infected cells. However, HIV-specific CD8 T cells eventually get exhausted and subsequently dysfunctional (49), or immune escape by the virus occurs due to mutational changes (50). As patients move into the chronic, or asymptomatic, phase of infection, there is continued loss of CD4 T cells and accumulation of residual inflammation. Despite suppressive ART, immune abnormalities and chronic inflammation persist, and people living with HIV do not have an immunological profile equivalent to persons without HIV infection (51-53). The underlying immune perturbations and low-grade chronic inflammation contribute to AIDS-related co-morbidities (54). In the modern ART era, co-morbidities including cardiovascular disease, cancer, osteoporosis, neurocognitive disorders, thrombosis and pulmonary diseases are thought to be driven by HIV-induced chronic inflammation. Indeed, elevated levels of interleukin (IL)-6, C-reactive protein and soluble monocyte activation marker CD14 are predictors of increased mortality in people living with HIV (55-58). Some of the immune

changes and abnormalities occurring during the acute, chronic and AIDS phases of HIV infection are shown in Figure 4.

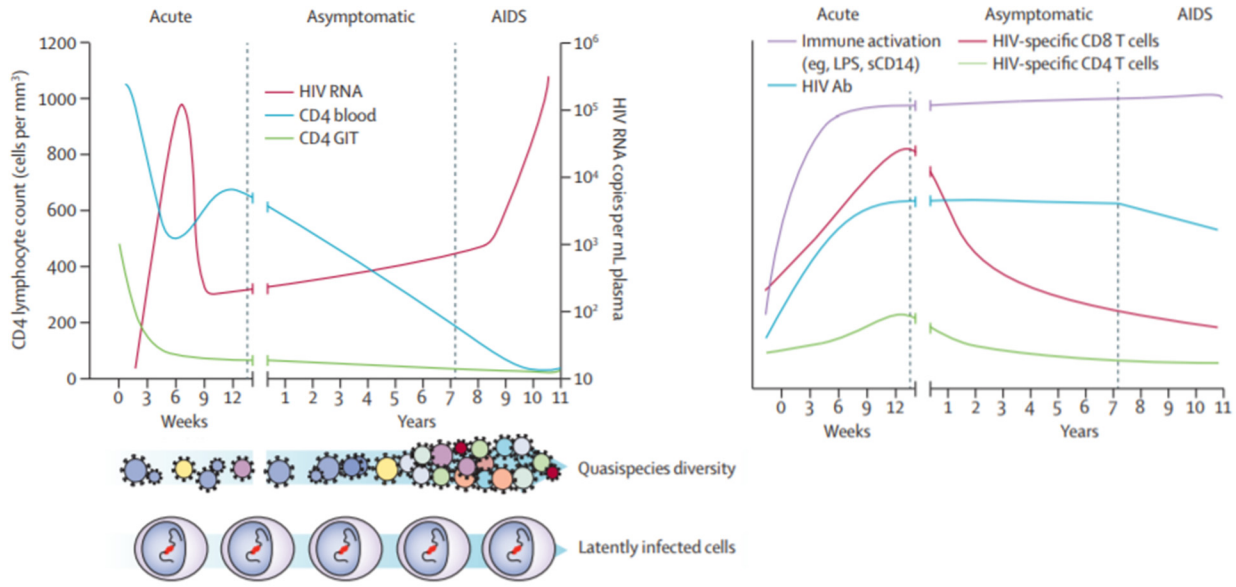

Figure 4: Pathogenic history of untreated HIV-infected patients as they go through the acute, chronic and AIDS phases of HIV infection (42).

1.1.5 Antiretroviral therapy (ART)

Individuals living with HIV in the present era have a life expectancy close to that of HIV-uninfected individuals thanks to ART (59, 60). Among those living with HIV in the world in June 2017, 20.9 million have access to ART, which is almost three times the number in 2010 (16). The increase in the prevalence of HIV may be explained by the increase in ART access: indeed, HIV-infected people are living longer after ART initiation (61). The underlying principle of ART is to target the different receptors, enzymes and viral proteins involved in the HIV life cycle in order to achieve a suppressed viral load. While early treatment strategies focused on opportunistic infections in the context of HIV/AIDS and disease management, the introduction of combination ART and the discovery of viral protein inhibitors greatly improved treatment efficacy. The idea of combining different antiviral drugs within an ART regimen was conceived to limit the emergence of drug resistance (62). Nevertheless, studies conducted in Western and Northern Europe has

shown that newly developed drugs and efficacious regimens, coupled with excellent patient adherence, can reduce the emergence of drug resistance (63, 64).

Drugs that have been approved in the treatment of HIV/AIDS can be divided into six types. Nucleoside reverse transcriptase inhibitors act through viral DNA chain termination by preventing bond formation with incoming nucleoside triphosphates during DNA synthesis (65). Non-nucleoside reverse transcriptase inhibitors, on the other hand, bind HIV-1 reverse transcriptase to induce a conformational change within the site of substrate binding, thus limiting enzymatic activity (66). Integrase inhibitors are molecules that target the strand transfer reaction that inserts HIV-1 DNA to the host genome (67). Protease inhibitors prevent the activity of the HIV-1 protease that cleaves Gag and Gag-Pol precursor proteins through mimetic mechanism or by preventing enzyme dimerization (68). Fusion inhibitors act by mimicking one of the two domains of the viral gp41 protein that interact with each other during viral fusion to the host cell, thus preventing the interactions required for virus entry (69). Finally, CCR5 antagonists work by binding to the transmembrane domains of the chemokine receptor to induce a conformational shift that goes unrecognized by the envelope of HIV-1 (70).

For an ART-naïve person infected with HIV, the ART regimen used during initiation consists of two nucleoside reverse transcriptase inhibitors and a third drug from the three other inhibitor categories coupled with a pharmacokinetic booster (71). For most individuals, the viral load usually becomes undetectable within 12 to 24 weeks of treatment. Factors predictive of virological success include low baseline viremia and high potency ART regimen coupled with excellent adherence (71). Although modern ART is very effective and generally well-tolerated, it is not without its disadvantages. Indeed, it requires excellent adherence in order to be maximally beneficial (72). Furthermore, ART is very costly, which makes it difficult for all persons living with HIV to access it, especially in low-income regions such as sub-Saharan Africa where HIV is in epidemic proportions (73).

1.2 VIRAL LATENCY AND HIV RESERVOIRS

1.2.1 Establishment and maintenance of viral latency

Despite intensive research to understand the pathogenesis of HIV infection and the development of therapeutic strategies, HIV/AIDS remains an incurable disease due to the establishment of viral latency within cellular or anatomical sanctuaries termed reservoirs. Viral latency is established very early in the acute phase of the infection (74), wherein HIV persists in reservoirs as replication-competent proviral DNA that remains transcriptionally dormant within the host chromosomal DNA following integration (75). This enables HIV to effectively escape host immune defenses since the latter do not detect any viral antigens. While the integrated provirus lacks the capacity to express viral RNA and viral proteins, it retains the ability to resume its replicative activity following treatment interruption (76). Even during long-term suppressive ART, the viral reservoir is not purged, especially in tissue compartments that are exposed to sub-therapeutic concentrations of antiretroviral drugs (77). When ART is initiated during primary infection and continued for greater than 10 years, the HIV reservoir may be reduced, although not eliminated (78).

Various processes were proposed to contribute to the maintenance of viral latency. One mechanism that is currently strongly debated is the presence of continuous low-level viral replication that replenishes the pool of latently infected cells despite treatment. Indeed, evidence of ongoing viral replication has been observed in patients under long-term suppressive ART (79, 80), perhaps favored by cell-to-cell transmission of HIV (81), in addition to trace amounts of residual viral RNA in the blood plasma (82, 83). However, these observations have been challenged with contradictory findings demonstrating a lack of genetic evolution of HIV-1 that should arise through viral replication (84, 85). Another means of maintaining HIV latency may be through homeostatic cellular proliferation, wherein the provirus integrates within or near regions of the host genome involved in cell cycle processes, resulting in the expansion of latently infected cells (86, 87). Once again however, this theory has been challenged as there is no evidence

indicating that the integrated provirus is replication-competent (88). The silencing of the integrated viral DNA itself can be maintained through mechanisms such as epigenetic histone modifications that suppress viral gene expression (89), the orientation of the integrated provirus within the host genome that either allows for viral promoter occlusion by a host polymerase (90, 91) or convergent collision between host and viral polymerases resulting in premature termination (92), and the cytoplasmic sequestering of transcription factors in resting cells (93).

1.2.2 Cellular reservoirs of HIV

Latently infected memory CD4 T cells are the most well-documented HIV reservoirs to date (1), and this cellular sanctuary of the virus will be discussed in more detail below. Macrophages and myeloid cells may also act as reservoirs. Indeed, viral species were found in microglia (94), rectal macrophages are distinctly permissive to HIV-1 (95), vaginal macrophages exhibited significant viral replication (96), and alveolar macrophages experienced very low levels of cell death as opposed to interstitial macrophages during SIV infection (97). Astrocytes are another proposed viral reservoir, as integrated viral DNA has been detected in this type of cells in HIV-infected individuals (98).

1.2.3 Anatomical reservoirs of HIV

Various anatomical sites in the human body have developed semi-permeable barriers and unique immunological landscapes as mechanisms of protection against foreign pathogens. While such measures have beneficial goals, the seeding of these sanctuaries by HIV or other infectious agents confers them detrimental characteristics. Indeed, due to restricted access to these sites, HIV is able to thrive in these compartments and establish viral latency. These anatomical reservoirs have thus become important topics of research in the quest to find a cure to HIV. One such reservoir is the central nervous system. The central nervous system is separated from the circulatory system by the blood-brain barrier. The latter is made of cells that create a tight and selective barrier restricting the passage of compounds from blood vessels in order to prevent

various neuropathologies (99). These restricted substances include antiretroviral drugs that experience poor penetration to nervous sites (100). Since HIV can be found in the brains of HIV-infected individuals as early as the acute phase of the infection (101) and trace amount of infection occurs despite ART (102), the virus can establish viral latency in cells of the brain. HIV also finds sanctuary in testicular tissue. To ensure the proper development of spermatocytes, the male genital tract and the testes possess barriers that restrict the influx of substances from the blood, like the central nervous system. Hence, antiretroviral drugs similarly experience hurdles to access these sites and end up at ineffective concentrations in the seminal fluid (103). The testes contained differential viral load in semen compared to plasma suggesting a distinct compartmentalization of the virus within seminal fluid (104), detectable HIV RNA levels even when adequate antiretroviral penetration was achieved (105), and HIV-specific CD8 T cells (106). Furthermore, a study examining bilateral testes in both HIV^+ and HIV^- individuals has demonstrated that the testes contain T cells that are more permissive to HIV, that exhibit a highly activated phenotype and that have suppressive potential against HIV-specific CD8 T cell activity (107), which further lends support for the persistence of HIV in testicular tissue. Sub-therapeutic levels of ART within these reservoir sites may contribute to the emergence of drug-resistant mutations and transmission of drug-resistant HIV variants. Furthermore, higher genital HIV RNA level increases one's risk for heterosexual transmission, independently from blood plasma (108). Other anatomical reservoirs of HIV are the lymph nodes. Lymph nodes contain germinal centres wherein B cells acquire important functional characteristics through interactions with dendritic cells and T follicular helper cells. T follicular helper cells were shown to be the primary central memory CD4 T cell subset that is susceptible to HIV (109) and contained replication-competent HIV DNA (110). Since B cell follicles are sites where the humoral immune response is built, CD8 T cells, specifically those with normal killer functionality, have restricted access to these sites (111), and B cell follicles within the lymph nodes can serve as important anatomical reservoirs where HIV establishes viral latency as a result of hindered anti-HIV immune responses.

1.3 T CELL SUBSETS AS POTENTIAL CELLULAR RESERVOIRS OF HIV

1.3.1 Review of T cell development

T cells constitute a group of specialized blood cells that arise from a process known as hematopoiesis. During development, cells derived from the mesoderm of the embryo are conferred a hematopoietic fate through induction by growth factors (112). Subsequently, there are two waves of hematopoiesis: embryonic hematopoiesis, in which red blood cells are produced to oxygenate embryonic growth, and adult hematopoiesis, when hematopoietic stem cells arise to differentiate into various immune cells (113, 114). Hematopoietic stem cells, which reside in the bone marrow (115), undergo an initial lineage commitment process by producing common lymphoid progenitors (116) and common myeloid progenitors (117). The former gives rise to lymphocytes and natural killer cells while the latter gives rise to megakaryocyte-erythrocyte or granulocyte-macrophage progenitors. Lymphoid progenitors may also arise through a different pathway involving multipotent progenitors (118). This subset of hematopoietic stem cells can express genes associated with the induction of T cells and B cells. The development of T cells occurs in an organ called the thymus, which has a microenvironment that facilitates the generation of a diverse T cell repertoire (119). A small proportion of common lymphoid progenitors and lymphoid-primed multipotent progenitors settle in the thymus following migration from the bone marrow. These progenitor cells travel inside the thymus and establish in the endothelium with the help of various homing molecules (120-122). Once established, the process of T cell differentiation begins.

Thymic-settling progenitor cells lack the expression of both CD4 and CD8, and are therefore coined double negative (DN) cells. These DN cells are further divided into four subgroups based on their expression of CD25, which is part of the IL-2 receptor, and CD44, an adhesion molecule involved in cell migration. DN1 cells ($CD25^{-}$ $CD44^{+}$), which are also called early thymic progenitors, can give rise to T cells, natural killer cells, dendritic cells and B cells, as well as cells of the myeloid lineage (119, 123). Eventually, DN1 cells will gradually lose their ability to

differentiate into non-T cell populations through the actions of thymic epithelial growth factors, receptor ligands and signaling pathways to become DN2 cells ($CD25^+$ $CD44^+$) (124). DN2 cells subsequently migrate through the cortex of the thymus, while in the mean time lineage restrictions and elimination of non-T cell fates continue (125). Gene rearrangement at the T cell receptor (TCR)-β, TCR-γ and TCR-δ loci begins (126), a mechanism that is completed within the DN3 stage. Indeed, among DN3 cells ($CD25^+$ $CD44^-$), upon correct VDJ recombination of *Tcrb* and subsequent expression of TCR-β, the latter can form a functional pre-TCR complex with CD3 and pTα (127). This process is called β-selection, since cells with unsuccessful TCR-β expression undergo apoptosis (128), and gives rise to αβ T cells. In contrast, γδ T cells result from correct rearrangements of TCR-γ and TCR-δ (129). By the end of the DN3 stage, all cells are committed to the T cell lineage. DN4 cells ($CD25^-$ $CD44^-$) upregulate CD4 and CD8 to yield double positive (DP) cells, notably through pre-TCR signaling (130). The rearrangement of the TCR-α chain further confers the DP population with a non-specific yet functional TCR-αβ complex, and a low affinity interaction between the newly-formed TCR and self peptide-bound MHC molecules found on cortical epithelial cells, dendritic cells and fibroblasts enables DP cells to survive and differentiate (131). This mechanism is called positive selection. DP cells also interact with self-antigens presented by medullary epithelial and dendritic cells, undergoing apoptosis if the interaction occurs with high avidity (131). This process is termed negative selection, since autoreactive T cells that can cause autoimmune damage are eliminated.

The mechanism for transitioning from a DP phenotype to a single positive (SP) CD4 or CD8 phenotype has been described using several models. In one model, either CD4 or CD8 is downregulated through the termination of CD4 or CD8 gene expression. MHC specificities come into play, as SP cells are rescued if the MHC matches the lineage (MHC class I for CD8 thymocytes and MHC class II for CD4 thymocytes) and eliminated otherwise (132). However, a more recent study has shown that MHC class II-restricted thymocytes are not always rescued towards the CD4 lineage and instead follow the CD8 lineage (133). In another model, a weak TCR signal terminates *Cd4* gene expression and produces a CD8 T cell, whereas a strong TCR signal terminates *Cd8* gene expression and produces a CD4 T cell (134). This model has been updated with a duration

determinant, wherein a short TCR signal produces CD8 T cells and a long TCR signal produces CD4 T cells (135), as well as a kinetic component, wherein the *Cd8* gene is initially terminated in DP cells and the consequence on TCR signaling is assessed (136). If signaling persists in the absence of the CD8 molecule, the cells become CD4 thymocytes. If the signaling does not persist, the cells differentiate into CD8 thymocytes following restored CD8 expression.

1.3.2 Phenotypic and functional characteristics of memory CD4 T cells

Cells with a memory phenotype constitute a subset of CD4 T cells that arise from surviving effector cells following an immunological threat. Upon antigenic exposure by antigen-presenting cells and concomitant cellular activation, effector CD4 T cells are mobilized through proliferation and differentiation, migrate to the site of inflammation and respond to the threat. Once their effector functions carried out, about 90% of the primed cells undergo apoptosis; the surviving cells become long-lived quiescent memory CD4 T cells (137). Memory CD4 T cells have the potential to quickly proliferate and regain their effector functionality upon secondary exposure to antigens. Studies examining the maintenance of lineage-specific functions in memory CD4 T cells have suggested that distinct epigenetic modifications and transcriptional mechanisms found in memory cells may play a critical role in the construction of a rapid, robust and heterogeneous response in future antigenic challenge (138). Compared to naïve cells, memory CD4 T cells produce a more efficient response to antigenic re-challenge and differentiate into more potent effector cells. Meanwhile, compared to effector cells activated during primary exposure, they preserve the ability to induce cellular proliferation and are less susceptible to cell death (139). While most memory CD4 T cells circulate through blood and lymphatic vessels towards target tissues, studies have shown that a portion of memory cells coined as resident may not re-enter circulation and are instead retained by various types of tissues (140).

Based on their proliferative and functional potential, memory CD4 T cells were first classified into a central memory subtype and an effector memory subtype: whereas the former resides in lymph nodes and can replenish the effector cell pool, the latter migrates to inflamed

sites and can mount rapid immune responses (141). More recent research has shown that memory CD4 T cells may not exist in a dichotomy. Indeed, other CD4 subsets arising during differentiation have been identified as showing a memory phenotype (142, 143). These include memory subsets such as stem cell memory T cells, central memory (CM) T cells, effector memory (EM) T cells and terminally differentiated (TD) T cells, each differentiating from the previous one (144). Table 1 shows markers that phenotypically characterize T cells at various stages of differentiation. Beyond a differentiation status perspective, memory CD4 T cells have also been classified based on their helper functions. For instance, Th1 cells participate in protective measures against bacteria, Th2 cells provides immunity against parasitic species and Th17 cells are involved in host immune defense mechanisms against extracellular bacterial and fungal threats (145).

	CCR7	CD62L	CD45RA	CD27	CD28
Naïve cells	+	+	+	+	+
T_{CM} cells	+	+/−	−	+	+
T_{TM} cells	−	+/−	−	+	+/−
T_{EM} cells	−	+/−	−	−	+/−
T_{EMRA} cells	−	−	+	−	−

T_{CM}, central memory; T_{EM}, effector memory; T_{TM}, transitional memory; T_{EMRA}, $CD45RA^{+}$ T_{EM} cells.

Table 1: Phenotypic markers identifying T cell subsets (146)

Memory CD4 T cells are particularly important in the mounting of an effective response against viral infections. Once the host organism encounters a virus, either through pathogenic exposure or immunizing vaccines, the immune system triggers a cascade of events that transform naïve CD4 T cells into effector CD4 T cells and finally generates memory CD4 T cells. As discussed above, these cells with a memory phenotype have a greater and more efficient potential in subsequent exposure to virus in terms of response potency and cellular recruitment. Long-lived memory CD4 T cells may play a crucial and protective role in secondary viral infection. For instance, upon re-infection with the influenza virus, the lungs, which already benefit from greater production of inflammatory cytokines and chemokines triggered by memory CD4 T cells, also experience a rapid onset of innate protection mediated by these cells (147, 148). Not only do the cells have the capacity to respond effectively to re-infection and reduce the viral load, but

importantly they can do so at earlier stages of pathogenesis. In a primary infection, the virus can infect cells and replicate with impunity until enough viral replication cycles have occurred to trigger an immune response. The early activation of memory CD4 T cells could reduce the damage done by the virus at low viral loads. Adding to this the fact that memory CD4 T cells work independently of pathways recognizing pathogen-associated molecular patterns (PAMPs) (147), this gives an advantageous edge to the host since viruses can actively inhibit the antiviral pathways that recognize double-stranded RNA by restricting the enzymes involved in them (149). Memory CD4 T cells also have the capacity to travel to B cell follicles more quickly through the higher expression of B cell follicle homing receptor CXCR5, giving them the ability to activate in an accelerated manner naïve B cells, which then class switch to produce relevant immunoglobulin isotypes (150). T cell-activated B cells can then produce functionally diverse antibodies against viruses in the early phases of infection.

1.3.3 Memory CD4 T cells during HIV infection

In the context of HIV infection, the progressive depletion of CD4 T cells is one of the key features of the disease's pathogenesis profile. As such, much research has gone into the study and understanding of the mechanisms underlying CD4 T cell death, namely how the virus weakens CD4 T cells' survival capacity (151) and how the immune system also contributes to cellular suicide through innate responses (152). One critical question asked by scientists was whether CD4 T cells are preferential targets of HIV or not. Indeed, determining the predispositions that enhance viral effects on CD4 T cells, potentially supporting their loss, may enlighten the pathways underlying HIV pathogenesis at the cellular level. That HIV-specific memory CD4 T cells were selectively infected by the virus was first shown around 15 years ago (153-155). In one of the first study examining this phenomenon, Douek *et al*. recruited 12 HIV^+ adults comprised of acutely and chronically infected individuals as well as treated and untreated individuals. After sorting HIV-specific memory CD4 T cells and cytomegalovirus-specific memory CD4 T cells based on the production of interferon (IFN)-γ due to antigen stimulation, as well as unstimulated memory CD4 T cells, they observed that the HIV-specific subset contained higher

levels of HIV DNA compared to the other two cell populations. Upon examining p24 expression in CD4 T cells infected *in vitro*, they found successful infection to successively more cells after each round of cell division and greater frequency of activated HIV^+ naïve CD4 T cells. This observation was in line with previous findings showing that activated cells were more susceptible to infection than quiescent cells in culture conditions (156). Thus, they explained the greater levels of HIV DNA in HIV-specific memory CD4 T cells to the fact that HIV antigens activate the naïve subset, making it preferentially susceptible to infection. These naïve cells expand to effector and finally memory cells, creating a pool of HIV DNA-harboring memory CD4 T cells. Another explanation may be due to the greater expression level of the HIV co-receptor CCR5 found on memory CD4 T cells. Indeed, studies published before (157-159) and after (160, 161) the one by Douek *et al*. have shown that this co-receptor that provides a measure of the permissiveness of cells to HIV was upregulated and predominantly present among memory CD4 T cells. Therefore, the preferential infection of memory CD4 T cells could be due to the greater potential for the virus to enter the cells during the early phase of infection thanks to the expression of CCR5 on the cell surface.

1.3.4 Persistence of HIV in effector memory and memory $CCR6^+$ CD4 T cells

The persistence of HIV within successfully ART-treated patients was proposed to be a result of latent virus in CD4 T cells. While most HIV DNA were present in an unintegrated replication-deficient form, a minuscule portion of CD4 T cells harbored integrated replication-competent viral DNA (162) that do not produce viral proteins (75). These latently infected CD4 T cells can arise despite early ART initiation (163) and remain unhindered by the treatment for a period spanning most of the human lifespan (164). The establishment of a cellular reservoir of HIV in specific subsets of memory CD4 T cells was first shown by Chomont *et al*. In their study (1) comprised of HIV-infected individuals under suppressive ART, some of which were included before and after ART initiation, they found a drop in viral replication after long-term ART, with a concomitant integration of HIV DNA within the host DNA of memory CD4 T cell subsets equal to the total viral DNA detected. Furthermore, in patients with plasma viremia levels below the

threshold of ultrasensitive quantification, CM, transitional memory and EM CD4 T cells contributed the most and in that order to the size of the latent reservoir and produced replication-competent virus upon co-culture with uninfected allogeneic dendritic and CD4 T cells. Thus, this study identified a crucial immune cell subset that participates in the existence of the long-lived HIV reservoir. Indeed, memory CD4 T cells, which themselves are endowed with inherent survival and proliferative capabilities, can replenish the HIV reservoir despite ART initiation and harbor viral material that can infect surrounding cells upon ART cessation. More recent studies have lent support to the characterization of effector memory CD4 T cells as a cellular reservoir of HIV. In one study where the authors developed a new RNA fluorescence *in situ* hybridization-flow cytometry assay capable of detecting viral RNA in individual cells, they found that effector memory CD4 T cells exhibited evidence of viral transcription activity based on the presence of HIV RNA inside the cells of both ART-naïve and ART-treated patients (165). Another study used a high-throughput sequencing assay in conjunction with comparative genomics to sequence HIV proviruses and evaluate their replication competency. The authors demonstrated that effector memory CD4 T cells harbored the highest levels of potentially replication-competent, genetically intact proviral DNA in HIV-infected individuals under long-term suppressive ART (166).

Three additional cell subsets within the memory CD4 T cell population characterized by their functional activity have been proposed as potential cellular reservoirs of HIV. These latently infected subsets express the chemokine receptor CCR6, which is involved in Th17 polarization, and consist of total memory $CCR6^+$ CD4 T cells, Th1Th17 CD4 T cells and memory $CCR6^+CCR4^-CXCR3^-$ CD4 T cells. When investigating the permissiveness and resistance to HIV infection of functional subsets of memory CD4 T cells, cells expressing CCR6 were found to be highly enriched in integrated proviral species in ART-naïve people living with HIV, with Th1Th17 cells being greatly permissive to HIV (167). This last memory CD4 T cell subset is characterized by the co-expression of CCR6 and CXCR3, the latter being involved in the homing of cells to inflammatory sites. These findings were substantiated by the same team as they showed through transcriptional profiling that Th1Th17 exhibit molecular signatures associated with HIV permissiveness, cellular activation

and antiviral activity (168), and that all memory CD4 T cells expressing CCR6, including the three mentioned above, are depleted in peripheral blood and greatly susceptible to infection by HIV (169), and contain high levels of replication-competent viral DNA (170). While HIV-1 sequences persisted in Th1Th17 cells, the latter contributed inordinately at any time point as well as over time by harboring greater levels of total (171) and integrated HIV DNA (172). Meanwhile, memory $CCR6^+$ cells that lack the expression of both CCR4 and CXCR3 are the most numerous Th17-polarized CD4 T cells in the lymph nodes and peripheral blood of ART-treated HIV-infected individuals, and they were shown to harbor replication-competent integrated viral DNA (173). Together, these findings demonstrate the tremendous phenotypic and functional diversity of memory CD4 T cells that contribute to the long-lived HIV reservoir in various tissues during HIV infection.

1.3.5 Origins of double negative T cells

Double negative (DN) T cells are T cells that lack both the CD4 and CD8 surface molecules. As previously discussed, the T cell differentiation pathway ends in the thymus with single positive cells expressing either CD4 or CD8 molecules. Hence, DN T cells found in the periphery are hypothesized to undergo a different developmental mechanism, occurring either in the thymus or extrathymically. As such, three models have been proposed to explain the origin of DN T cells. In the first model, DN T cells arise by escaping the T cell maturation pathway in the thymus (174, 175), while the second model refutes the first model, stating that DN T cells do not arise by escaping selection but rather originate as a result of it (176). Of interest is the third model in which HIV plays an important role in the emergence of DN T cells. The subset is suggested to arise from CD4 T cells wherein the single positive cells downmodulate the CD4 molecule. This model is supported both by mice and monkey studies. In TCR-transgenic mice, high dosage of antigenic peptides decreased the levels of CD4 SP cells and stimulated the expansion of DN cells among thymocytes (177). Evidence for these observations was also found among the natural SIV host African green monkeys, in which CD4 T cells that experience antigenic stimulation acquired the memory phenotype and downregulated CD4 expression *in vivo* (178). In the context of HIV,

transcriptional products of viral replication during infection have been shown to be involved in the peripheral expansion of DN T cells. Indeed, DN T cells were found to arise as a result of the actions of HIV proteins that induce the downregulation of CD4 on CD4 T cells (179). Using co-precipitation assessments and immunohistochemistry in HeLa cell lines, it was found that cells that expressed CD4 alone were able to export the molecule to the surface. Meanwhile, cells that expressed both CD4 and the HIV glycoprotein precursor gp160 saw these two molecules form complexes that remained in the endoplasmic reticulum (ER), thus preventing CD4 trafficking to the plasma membrane (180). Another mechanism of HIV-induced CD4 downregulation before export from the ER to the plasma membrane involves the viral protein Vpu, a protein expressed at later stages of infection (181). Vpu acts on newly synthesized CD4 molecules in the ER, marking them for degradation by proteolysis through the ER-associated degradation (ERAD) pathway (182). Finally, once on the surface of T cells, CD4 molecules can be targeted by the viral protein Nef for endocytosis and degradation. In Nef-expressing VB cell lines and surface CD4 biotinylation experiments, CD4 migrated to the cell surface in normal levels but were subsequently degraded from the surface with a half-life four times less than in control cells (183). The viral protein essentially introduces CD4 to proteins that internalize the molecule for degradation through lysosomal mechanisms (184). Together, these findings suggest that HIV infection plays a significant role in the induction of DN T cells, which can in turn participate in host immune responses as well as HIV/AIDS pathogenesis.

1.3.6 DN T cells during HIV infection

In the context of HIV/AIDS, the frequency of γδ DN T cells were found to be higher in certain HIV^+ populations, including patients with co-infections and cancer (185, 186). As well, HIV RNA has been detected in αβ DN T cells from the peripheral blood and lymph nodes of HIV^+ patients (187). The findings demonstrating the enrichment of DN T cells in HIV/AIDS were both substantiated and challenged in recent studies. For instance, in patients living with HIV and who have reached the AIDS stage of disease progression, there was a substantial increase in the proportion of DN T cells in peripheral blood, so much so that while the DN T cell subset expressing

CD161 was twice as high in healthy individuals, the total frequency of $CD161^+$ DN T cells in peripheral blood was comparable between groups (188). In the female genital mucosa, a subset of DN T cells expressing the lymphocyte-activating gene (LAG)-3, a protein found in the mucosa at high levels, were greatly permissive to HIV (189). Meanwhile, in a cohort of recently infected individuals, DN T cells were observed to decrease in number in patients who reached later stages of HIV disease progression (190) and in patients who are immunological non-responders (191). Notwithstanding the variable changes in frequency during HIV infection, DN T cells play several roles in disease. In primary HIV infection, DN T cell proportions negatively correlated with CD8 T cell activation, suggesting the potential of DN T cells to predict and decrease immune activation (192). DN T cells may also contribute to specific and distinct responses to HIV, since contrasting TCR expression and cytokine production were observed between HIV-serodiscordant partners (193).

1.3.7 Persistence of HIV in DN T cells

One key area of research about DN T cells in the context of HIV/AIDS pertains to its contribution to the persistence of the virus despite treatment through the establishment of a cellular reservoir. In a study using magnetic cell sorting to isolate T cell subsets and polymerase chain reaction (PCR) assays to measure viral DNA and RNA, researchers found that while CD4 T cells harbor the bulk of viral genome, as expected, it accounts for a small portion of active transcription; instead, the latter occurs primarily in DN T cells in patients with viremia (194). Based on the expression of p24, another study, this time examining pleural sites during co-infection with HIV and tuberculosis (TB), posited that DN T cells were the main source of productive infection and HIV-1 in the membranous compartment (195). Due to enhanced replication competency of DN T cells, their effects on disease control, perhaps through the establishment of a latent reservoir, is an important area of research. In patients who failed to respond to ART, as opposed to HIV-infected responders, isolated DN T cells showed significant viral DNA integration (196). While the authors suggested that DN T cells may not be a major reservoir in patients on ART, the cell subset is nevertheless a source of virus if treatment is

terminated, thus contributing to HIV persistence. A more recent study investigated the expression of the Gag structural proteins, which are produced from integrated HIV genome. The paper showed that HIV proteins internalized CD4 T cells after infection, conferring the subset with a DN phenotype, and are expressed in patients on ART (197). While DN T cells can also produce viral proteins from replication-defective proviruses, they may also express replication-competent proviruses and release virus that can infect other cells, thus contributing to the establishment of a non-classical cellular HIV reservoir in ART-treated people living with HIV.

1.3.8 CD32a: a recently discovered and controversial cellular marker of HIV latency

In order to develop therapeutic strategies that can target cells in which HIV remains hidden, the discovery of cellular markers of viral latency could greatly facilitate this endeavor. In a study published last year (198), a team of scientists looked at the expression and upregulation of genes in *in vitro* HIV-infected quiescent CD4 T cells. Among the genes that are highly expressed in latently infected cells, the low affinity immunoglobulin G Fc fragment receptor CD32a was the most induced on these infected CD4 T cells (198). CD32a was not detectable on neighboring cells or cells that were productively infected with HIV-1 (198). Blood samples of ART-treated patients showed that only 0.012% of CD4 T cells express CD32a, yet these cells harbored HIV DNA copies and replication-competent proviruses (198). However, CD4 T cells expressing CD32 were found to be more activated and correlated with generalized immune activation in viremic, ART-treated patients and in healthy individuals (199). Moreover, another study observed that productively infected CD4 T cells from the peripheral blood of healthy donors expressing viral RNA and proteins upregulated both CD32 and HLA-DR, a marker of immune activation (165). These contrasting findings and the numerous studies presented at the 25th Conference on Retroviruses and Opportunistic Infections (CROI 2018) challenging the original paper are fueling the debate on the status of CD32a, and whether or not it is a marker of latently infected cells remains to be proven.

1.4 T CELL DYSFUNCTIONS DURING HIV INFECTION

1.4.1 T cell immune activation

Generalized immune activation is one of the hallmarks of HIV infection. High levels of immune activation are indeed observed in ART-naïve and, at lower degrees, in long-term ART-treated HIV-infected individuals and may result in immunological failure despite treatment (200, 201). In addition, immune activation may contribute to the establishment of viral latency by increasing the infection susceptibility in activated cells (1, 202). Early studies examining structural abnormalities of lymph nodes and blood samples of chronically infected patients found that CD4 and CD8 T cells expressed higher levels of the activation markers HLA-DR and CD38 (203). In addition, enrichment in HLA-$DR^{+}CD38^{+}$ CD8 T cells was linked to CD4 T cell depletion and disease progression (204). The establishment of a set point in immune activation during early infection predicted the rate of depletion of CD4 T cells (205). The persistence of pathological levels of immune activation in T cell subsets was associated with microbial translocation, higher levels of coagulation and inflammation (203). The mechanisms that cause immune activation are manifold. The first potential cause is HIV itself. Viral proteins Nef and Vpr are able to stimulate immune cells such as macrophages (206) and viral RNA can induce cytokine production through Toll-like receptors (207). Microbial translocation also plays an important role, as the loss of integrity of the gut epithelium allows for gut microbes to enter the body and further stimulate the immune system (208). Co-infections are additive factors that can provoke generalized chronic activation. Indeed, studies have shown that HIV-infected people who are co-infected with viruses such as Hepatitis C or human herpesvirus 8 have higher levels of T cell activation compared to HIV mono-infected individuals (209, 210). Immune senescence may also cause immune activation as aging cells produce high levels of inflammatory molecules both *in vitro* (211) and *in vivo* (212). Finally, an imbalance between the pro-inflammatory Th17 cells and anti-inflammatory regulatory T cells that inhibit other CD4 T cell subsets may influence the levels of immune activation (213).

1.4.2 T cell immune senescence and T cell immune exhaustion

While immune senescence is a contributor to increased immune activation seen during HIV infection, it is also itself a result of persistent and residual inflammation and chronic activation (214). HIV-induced immune senescence, through chronic activation, is a premature cellular aging process that may play a significant role in the pathogenesis of HIV and progression towards clinical AIDS (215). Studies have shown an accumulation of CD8 T cells that lack CD28 expression induced by the shortening of telomeres during successive rounds of replication (216). Viral replication and T cell activation indeed promote cellular proliferation and differentiation of cells that have encountered antigens. These T cells, especially CD8 T cells, eventually lose CD28, begin expressing CD57, and have a reduced capacity to produce IL-2 and to proliferate. Furthermore, their cytokine production profile becomes pro-inflammatory, with the secretion of IFN-γ, IL-1β and tumor necrosis factor (TNF)-α (217, 218). Meanwhile, another phenomenon occurs wherein increased HIV-induced cellular activation results in cellular exhaustion as T cells rapidly expend their functional resources as they try to respond to the infection. During chronic untreated infection, the programmed death (PD)-1 receptor was found to be highly expressed on the surface of exhausted antigen-specific CD8 T cells in mice and was associated with a pathway that impaired CD8 T cell function (219). In humans, the elevated expression of PD-1 on HIV-specific T cells was linked to cellular exhaustion and dysfunction, uncontrolled viremia and disease progression (220). CD4 T cells expressing PD-1 was also suggested to play a role in HIV persistence (221), while a subset of PD-1^{+} CD8 T cells co-expressing CD160 showed advanced dysfunction (49).

1.5 THE LUNGS AS ANATOMICAL RESERVOIRS OF HIV

1.5.1 Pulmonary immunity

The lungs are important anatomical reservoirs of HIV that have been relatively under-studied after the introduction of ART. To understand the mechanisms that confer the lungs with the capacity to provide HIV with a sanctuary for viral latency, the lungs' immunological landscape and specifically mucosal immunity must be considered. The lungs have the second most extensive mucosal surface in the human body after the digestive tract (222). The pulmonary mucosal surface is in constant contact with the exterior environment and thus with foreign agents, both organic and inorganic. To avoid underestimating or overestimating threats, the lungs understandably developed unique immune properties to protect the host, including surveillance functions monitoring harmless foreign agents and immune mechanisms against potential pathogens (222). Pulmonary immunity has both an innate aspect for broad and immediate responses, and an adaptive aspect for specialized responses.

1.5.1.1 Innate immunity within the lungs

The first line of defense within pulmonary innate immunity are defense mechanisms mediated by ciliated epithelial cells, periciliary fluid and mucus (223). These components allow for foreign agents to be captured prior to making their way to the lower respiratory tracts, be treated with antimicrobial substances and be propelled upwards to be swallowed or coughed out. The antimicrobial substances found in the periciliary fluid include peptides such as lactoferrin, which traps iron away from bacteria to prevent growth, lysozymes, which participates in the lysis of Gram-positive bacteria, C-type lectins, which can enhance phagocytic activity, and defensins, which act against bacteria, viruses and fungi (224). In addition to this humoral innate response, the lungs also experience a cellular innate response mediated by resident cells such as airway epithelial cells, dendritic cells and alveolar macrophages. Airway epithelial cells function against invading antigens by secreting antimicrobial peptides and identifying conserved antigenic

motifs known as PAMPs by using pattern recognition receptors such as Toll-like receptors (225). The secretion of antimicrobial peptides allows for the elimination of microbes and the recruitment of innate and adaptive immune cells, while recognition of PAMPs favors the expression of genes encoding for cytokines, chemokines and antimicrobial peptides (226). Lung resident dendritic cells also play an important role by serving an intermediate role between innate and adaptive immunity (227). Indeed, dendritic cells lining the respiratory tract have an increased ability to detect pathogens, migrate into draining lymph nodes, activate naïve T cells to become effector T cells, and initiate an adaptive immune response (228). Finally, alveolar macrophages are the most prominent cellular component of innate immunity in the respiratory lumen, as more than 90% of alveolar mucosal cells under normal conditions are alveolar macrophages (229). As such, alveolar macrophages serve as the primary phagocytes in pulmonary innate immunity. Their activities include release of proinflammatory cytokines and phagocytosis of foreign microorganisms and particulates (230), as well as maintenance of lung homeostasis and integrity (231). These functions require a certain degree of plasticity and phenotypic heterogeneity in order to address real pathogenic threats while limiting responses against self or harmless antigens (232).

1.5.1.2 Adaptive immunity within the lungs

The immune responses triggered during the innate phase of lung immunity is of significant importance in the initiation of pulmonary adaptive immunity. Indeed, studies have shown that following antigen binding of Toll-like receptors found on innate immune cells, the concomitant production of inflammatory signals promote T cell maturation and proliferation (233), and even enhance the activity of memory T cells during infection (234). Meanwhile, in the mucosa-associated lymphoid tissue, germinal centers form where antibody class-switching occurs on B cells that then migrate to the mucosa of the lungs to interact with various T cell and innate immune cell subsets (222). During viral infections, the lungs experience a substantial increase in the frequency of both CD4 and CD8 T cells (235, 236). As is the case in other instances of adaptive immune response, when the infection is over and the virus is cleared, long-lived

memory T cells remain in the event of a future re-infection. As the lung mucosa is a major site of pathogenic contact, a specialized subset of memory T cells coined tissue-resident memory T cells also establish a pool within the lung mucosa. These tissue-resident cells have enhanced abilities to respond more effectively to infection and promote survival (237). Thus, the lungs constitute a unique immunological effector site that maintains a dynamic microenvironment mediated by both innate and adaptive components before, during and after an immune challenge.

1.5.2 Pulmonary mucosal immunity during HIV/SIV infection

HIV infection is associated with higher rates of pulmonary clinical complications. The prevalence of chronic lung diseases, notably chronic obstructive pulmonary disease (COPD), among HIV-infected individuals exceeds that observed in age and sex-matched HIV-uninfected controls (238, 239). Bacterial pneumonias are up to 25 times greater in HIV-infected individuals with suppressed peripheral viral loads on ART compared to HIV-uninfected participants (240). Influenza is also a common respiratory illness in HIV-infected individuals on ART, even despite vaccination (241), and the incidence of TB is higher in HIV-infected individuals on ART than in the general population (242). Immune abnormalities present in the lungs of HIV-infected individual, namely inflammation, immune activation and immune senescence, were linked to the incidence of chronic lung disease, specifically COPD, which is present at 20-60% in HIV^+ persons compared to 7% in healthy people (3) and is the third leading cause of death in North America (243). Smoking, on the other hand, was not found to be effectual on the progression of HIV infection toward clinical AIDS (244), though it was linked to higher risk of mortality due to non-AIDS-related diseases (245) and smokers overall displayed decreased cytotoxic activity (246), reduced phagocytosis by HIV-infected alveolar macrophages (247) and increased HIV replication (248). Of particular interest, HIV infection is recognized as an independent risk factor of altered pulmonary immunity, independent of smoking status (238).

The lungs are highly perfused organs and HIV gains entry into the lungs by infecting peripheral blood lymphocytes that migrate towards the pulmonary compartment (249). Various

features of the pulmonary compartment may facilitate the dissemination of the virus. Due to the lungs' large alveolar surface area and very small diameter capillaries (250), abundant levels of pulmonary cells remain in close proximity to one another which may facilitate cell-to-cell transmission of the virus. Alveolar macrophages in particular can transmit the virus to T cells through antigen presentation (251), as they express higher levels of the intercellular adhesion molecule-1 in AIDS patients (252), as well as spread the virus through proliferation mediated by the higher release of granulocyte-macrophage colony-stimulating factor seen in HIV-infected individuals (253). In the acute phase of the infection, HIV uses the chemokine co-receptor CCR5 to infect host cells, while in the chronic phase of the infection, it enters the cell via attachment to CXCR4, whose expression is high in myeloid cells and increased in T cells in bronchoalveolar lavage (BAL) fluid of chronic AIDS patients (254). Of interest, the CCR5 tropic strain HIV-1_{Bal} was originally isolated from a lung (255). Despite contrasting findings on the magnitude of viral replication in the lungs of SIV-infected monkeys, with low levels during the acute phase of infection seen in some studies (256) and detectable viral RNA correlating with plasma viral load found in other studies (257, 258), these indicate a certain degree of SIV seeding and replication in the early stages of infection. Alveolar macrophages, which are found at the surface of the alveoli (259), may play a role in harboring persistent virus. Indeed, studies have shown that alveolar macrophages from BAL fluid collected from macaques were productively infected with SIV (260) and provided a sanctuary to the virus due to their low turnover rate in contrast to interstitial macrophages (97), which are found between the alveolar epithelium and vascular endothelium (259). It has been suggested that the significant levels of SIV RNA may be a causal factor in lung interstitial inflammation (261).

In terms of cellular changes following infection, there is a substantial influx of cytotoxic CD8 T cells, a phenomenon termed lymphocytic alveolitis, directed against alveolar macrophages that have been infected with HIV (262-264) and resulting in cough, dyspnea, interstitial pneumonitis, pulmonary dysfunctions (265), major inflammatory reactions (266) and other complications (267). Specific to the functionality of the lungs, the massive infiltration of cytotoxic CD8 T cells may cause structural damage, emphysema and restricted carbon dioxide diffusion

(268). Furthermore, these newly arriving CD8 T cells exhibit an exhausted phenotype and are functionally deficient (269). Meanwhile, CD4 T cells experience a reduction in their numbers due to their elimination by the incoming anti-HIV CD8 T cells, apoptotic dysfunction and attachment with alveolar macrophages (2). Pulmonary mucosal EM CD4 T cells displayed a Th1 and activated phenotype (270), but those with antigen specificity and IFN-γ and TNF-α secretion polyfunctionality were found in lower frequency in the BAL of HIV-infected individuals compared to healthy individuals (271). B cells experience an impaired behavior as they become activated non-specifically and produce less antigen-specific antibodies (272). This may result in imbalances in Th1/Th2 ratio and cytokine production (273) and dysfunctional formation of opsonizing antibodies (274), the latter in turn resulting in streptococcal pneumonia (275) and pneumococcal disease (276). Finally, alveolar macrophages, specifically the small ones, were found to be preferentially infected by HIV, which results in defective phagocytic capacity and potential infections of the lower respiratory tract (277). As a result of HIV infection, alveolar macrophages experience other abnormalities. They release cytokines and chemokines that promote viral replication and T cell proliferation, including pro-inflammatory molecules TNF-α, IL-1, IL-6 and IFN-α, they have impaired clearance capacity, and they are less prone to pathogenic response initiation due to the depletion of cells that release macrophage-activating cytokines such as IFN-γ (278) (Figure 5).

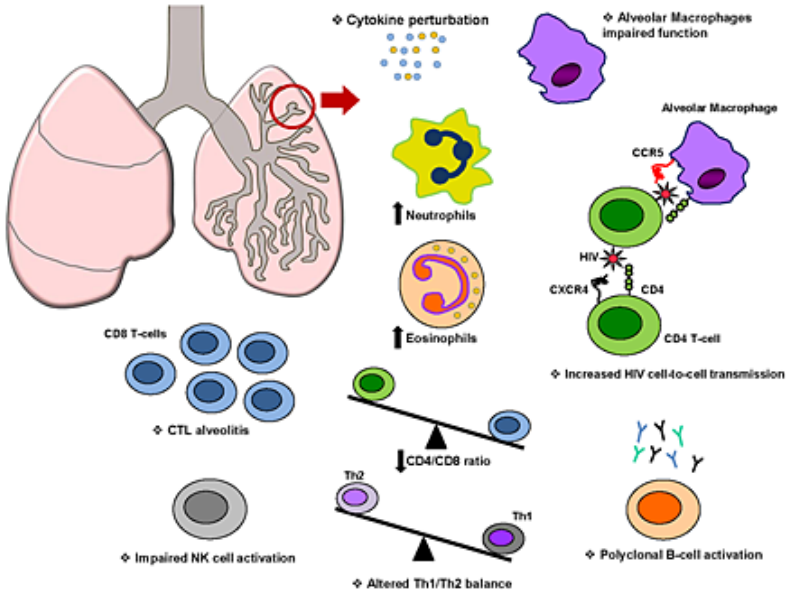

Figure 5: Immune abnormalities within the lungs during untreated HIV infection (2).

Interestingly, infection with a highly pathogenic chimeric simian-human immunodeficiency virus (SHIV) produced no significant longitudinal alterations to the microbiome of the lungs of cynomolgous macaques that had individual microbiotic variations (279). The bacterial flora of the lungs may therefore play a role in the pathogenesis and immune response to SIV or HIV, as previous reports have shown that SHIV infection resulted in low levels of viremia and CD4 survival (280). Studies on the pulmonary microbiome have also been done on humans with HIV, although to date no clear conclusions have been reached and the dynamics between the pulmonary HIV reservoir and the microbiome is uncharacterized (281-283).

1.5.3 Effects of pulmonary HIV co-infections on pulmonary mucosal immunity

Higher levels of HIV replication have been observed in various pathologies involving co-infectious agents including cytomegalovirus (284), *Pneumocystis jirovecii* (285) and *Mycobacterium tuberculosis* (286, 287). In the case of *Pneumocystis jirovecii* pneumonia for instance, HIV in cell-free BAL fluid was four times higher in co-infected patients (288), and co-culture of alveolar lymphocytes revealed that co-infected persons produced five times more virus (285). The mechanisms by which co-infections stimulate viral replication pertain to immune alterations in the lungs, stimulation of transcription and hindrance to host immune responses. For instance, opportunistic co-infections can activate latently infected alveolar macrophages into producing granulocyte-macrophage colony-stimulating factors that promote macrophage proliferation (253) and induce viral replication (289). Gram-negative bacteria may produce chronic activation through lipopolysaccharides, the major component of their outer membrane (290), which in turn induces the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) mechanism of HIV activation (291). Co-infections also have the ability to upmodulate CD4 molecules. As such, increased density of CD4, which is used by HIV during entry, effectively augments susceptibility to infection (292). Fungicidal activity (293) and neutrophil recruitment (294), both mediated by alveolar macrophages, were lower in co-infected populations. Of interest is TB, which is the primary cause of mortality in HIV-infected individuals worldwide even in spite of ART (295). Importantly, TB induces the expression of CCR5 and CXCR4, co-receptors

used by HIV for cell entry, thus promoting HIV infection (296). It was also associated with greater genetic variance in HIV (297) and enhanced viral replication in alveolar macrophages of people living with both HIV and TB (286, 287).

1.5.4 The lungs as anatomical HIV reservoirs in the ART era

After initiation of effective ART, partial immune reconstitution was observed in the lungs (298), though infections such as bacterial pneumonia are still more prevalent in HIV-infected individuals compared to healthy individuals (299). However, the specific effects of ART on pulmonary mucosal immunity and on viral latency in the lungs are not well documented, as most studies examining these correlates were conducted prior to the introduction of combination ART, during the era of mono- and dual-therapy (2). In addition, most of the studies performed to date have been conducted on individuals who were on ART for a relatively short time period (300), while those who examined HIV infection of alveolar macrophages did not use highly purified cell populations (301). In the ART era, there was a progressive reduction in several infectious diseases including cytomegalovirus disease, while co-infections such as *Pneumocystis jirovecii* pneumonia and TB experienced an increase in incidence (302). One longitudinal study, which recruited ART-naïve HIV-infected individuals in the post-ART era and examined BAL fluid before ART initiation, four weeks after initiation and 24 weeks after initiation, showed that the HIV burden and lymphocytic alveolitis decreased after treatment (300). Another study demonstrated CD4 T cell reconstitution following ART initiation (303), while residual HIV DNA persisted in CD4 T cells from BAL fluid despite six months of suppressive ART (300). Indeed, BAL cells harbored differential resistance mutations in the HIV reverse transcriptase compared to peripheral blood mononuclear cells (PBMCs), which is indicative of active viral replication in the lungs despite ART (304). Importantly, the anti-HIV polyfunctional capacities of lung mucosal CD4 and CD8 T cells remain unchanged following ART initiation (303).

In studies involving SIV-infected monkeys, the lungs were the anatomical compartment with the second highest levels of SIV RNA after lymphatic tissues (305). While ART allows for the

viral load in blood plasma to reach undetectable levels, low levels of viral replication continue to occur in anatomical sanctuaries, allowing HIV to mutate and evolve (306). Importantly, one monkey study using SIV Gp120-specific antibody labelling (immunoPET) and full-body imaging has found significantly detectable levels of SIV in the respiratory tissues of both viremic and ART-treated aviremic rhesus macaques (306). Genotypic studies examining HIV variants and phylogenies within the lungs compared to the peripheral blood have shown that the virus experiences a certain degree of compartmentalization in the pulmonary site. Indeed, HIV-1 lineages in alveolar macrophages independently evolved further from their ancestral lineage compared to blood monocytes (307), were phylogenetically apart from their circulating counterparts (308), and differentially used the chemokine co-receptors for entry into cells compared to virus in the blood (309). As discussed above, the lungs contain abundant levels of alveolar macrophages as well as lymphocytes in close proximity. This allows for greater potential for cell infection and active cell-to-cell transmission of HIV. In addition to harboring cells susceptible to infection and diverse HIV variants, the lungs also experience differential penetration depending on the antiretroviral drug used. Hydrophilic antiretroviral drugs are often administered in liposomal capsules since drugs with higher lipophilic potential penetrate the lungs better, and this was substantiated by the fact that efavirenz, which has high lipid solubility, had a higher potential of HIV RNA clearance in the pulmonary compartment (310). As seen in other anatomical reservoirs, drug penetration has critical ramifications in the establishment of viral latency. Thus, drugs with lower lipid solubility and consequently lower penetration can allow the lungs to become anatomical sanctuaries for the virus. However, to date, the clinical outcomes resulting from differential antiretroviral penetration within the lungs of ART-treated individuals has not been assessed. In addition, pulmonary mucosal T cell immunity in HIV^+ individuals under long-term suppressive ART has not yet been fully described. Therefore, the quantification of the HIV reservoir in pulmonary mucosal CD4 T cell subsets and their phenotypic characterization are of paramount importance to understand the dynamics governing viral latency and persistence in the lungs and develop targeted therapeutic strategies to purge the pulmonary anatomical reservoir. In addition, the cytotoxicity and anti-HIV specific responses of pulmonary CD8 Tcells

and their contribution to HIV persistence within the lungs of HIV^+ individuals under suppressive ART remains to be explored.

CHAPTER 2: METHODOLOGY

Author contribution: The methodologies presented in this chapter were designed by Syim Salahuddin, Dr. Cecilia Costiniuk, Dr. Mohammad-Ali Jenabian and Dr. Nicolas Chomont.

2.1 Study population and specimen collection

A total of 24 HIV-infected and 8 healthy participants were recruited at the McGill University Health Centre (MUHC) in Montreal, Quebec, Canada. HIV^+ participants were all ART-treated with suppressed plasma viral load for at least 3 years and a CD4 count of at least 350 cells per mm^3. Both HIV^+ and HIV^- participants were without any active respiratory symptoms, acute pulmonic process or pulmonary co-infections. Participants with contraindications to bronchoscopy or on medication with increased risks of bleeding were excluded. All participants underwent spirometric testing to ensure the absence of any undiagnosed obstructive pulmonary disease. Information on participant characteristics and antiretroviral drugs was extracted from the clinical database and participant electronic records. All participants consented to providing BAL fluid, pulmonary biopsies and peripheral blood, despite no medical necessity for the procedure. Bronchoscopies were performed by a trained respirologist at the MUHC Centre for Innovative Medicine. A bronchoscope was passed through the mouth into the right lung, and a saline solution was squirted and collected to obtain 50 to 100 ml of BAL fluid. Bronchial biopsies were taken from the sub-segmental and segmental carinae of the right middle and right lower lobes. In addition, 40 ml of matched peripheral blood was also drawn.

2.2 Ethical considerations

This study using BAL fluid, pulmonary biopsies and peripheral blood from HIV-infected and healthy study participants was conducted in compliance with the ethical principles included in the Declaration of Helsinki and received approval from the Institutional Review Boards of the MUHC (#15-031), Université du Québec à Montréal (#602) and Centre Hospitalier de l'Université de Montréal (CHUM) Research Centre (#15-180). All study participants signed a written informed consent for their study participation.

2.3 Sample processing

Within 1 hour following the bronchoscopy, pulmonary biopsies were conserved at -80°C as dry pellets. The BAL fluid was centrifuged and pulmonary mucosal cells were obtained. The supernatant was conserved at -80°C to measure the levels of HIV RNA in cell-free BAL fluid. 0.5 to 1 million pulmonary mucosal cells were conserved at -80°C as dry pellets for HIV DNA and RNA quantifications. The rest were stained for phenotypic characterization and cell sorting by flow cytometry. The peripheral blood was centrifuged to obtain plasma, which was then conserved at -80°C. PBMCs were isolated by Ficoll density gradient centrifugation and subsequently stained for phenotypic characterization and cell sorting by flow cytometry (Figure 6).

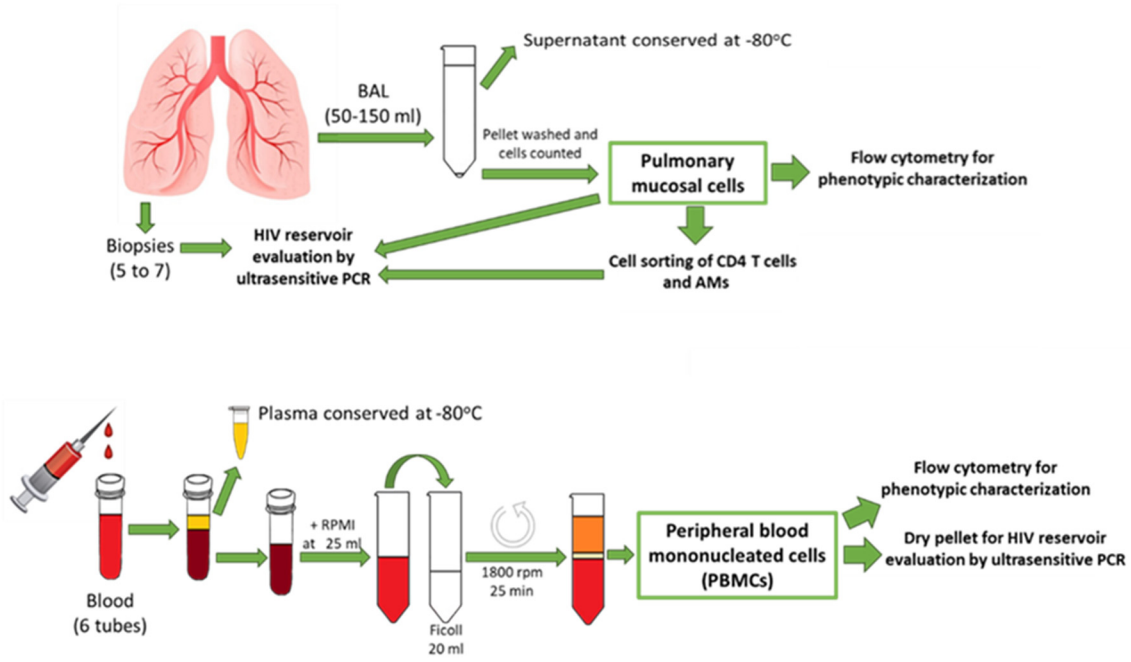

Figure 6: Isolation of pulmonary mucosal cells and PBMCs, and subsequent usage. “Alveolar macrophages” is abbreviated as “AMs”.

2.4 Cell sorting

A fraction of freshly collected cells from the BAL fluid were used for cell sorting of pulmonary CD4 T cells and alveolar macrophages. BAL cells were stained with CD3 AlexaFluor700,

CD4 FITC, CD8 APC-H7 and CD206 PE. Pulmonary $CD3^+CD4^+CD8^-$ T cells and $CD206^+$ AMs (277) were FACS-sorted on a BD-Aria cell sorter to obtain highly pure populations for HIV DNA and RNA quantifications. Of note, due to the limited quantities of cells recovered from the BAL fluid, these measurements were not performed in all participants.

2.5 HIV DNA and RNA quantifications

The frequency of cells harboring total HIV DNA (copies per million cells) was measured by Dr. Nicolas Chomont's lab at the CHUM Research Centre using a well-established assay (sensitivity of 1 copy per PCR reaction) (1, 311) with minor modifications to the original protocol. Notably, DNA from PBMCs and BAL cell pellets was extracted using the QIAamp DNA mini kit (Qiagen) before being subjected to PCR amplification. Cell-associated HIV RNA was quantified by ultrasensitive real-time (RT)-PCR as described in a previous study (312). CD4 T cells from PBMCs were purified by negative selection magnetic isolation.

2.6 Phenotypic characterization of T cell subsets

Multi-color flow cytometry staining was performed on freshly isolated PBMCs and pulmonary mucosal cells. A viability dye kit from Invitrogen was used to exclude dead cells from the analysis. Frequencies of naïve, CM, EM, TD and senescent T cells were measured on live CD4 T-cells by CD28, CD45RA and CD57 expressions. Regulatory T cells (Tregs) were defined as $CD127^{low}CD25^+FoxP3^+$. The expression levels of the immunosuppressive ectonucleotidases CD39 and CD73 were assessed. Th subsets were determined by CCR4, CCR6 and CXCR3 expression. Activated cells were identified with activation markers HLA-DR and CD38. Expression of HIV co-receptor CCR5 was assessed on CD4 T cells, while expression of PD-1, CD160, Granzyme B and perforin were assessed on CD8 T cells. Finally, CD32a and the associated IgG2b, κ isotype control were also used to determine the expression of the low affinity type II Fcγ receptor. A 3-laser BD Fortessa-X20 was used for acquisition and results were analyzed by FlowJo V10.2.

2.7 Statistical analyses

GraphPad Prism V6.01 was used for statistical analyses. Results in the text are presented as mean ± standard deviation. The Kolmogorov-Smirnov normality test was used to assess the Gaussian distribution. A paired Student's *t*-test was used to compare study variables when the paired values passed the normality test. A Wilcoxon matched-pairs signed rank test was used for comparison when the paired values did not pass the normality test. Non-paired comparisons between study groups was conducted by the Mann-Whitney *U* test. Spearman's rank correlation coefficient was computed for correlation analyses.

CHAPTER 3: RESEARCH FINDINGS

Author contribution: The experiments in this chapter were conducted by Syim Salahuddin, Dr. Omar Farnos and Amélie Pagliuzza. Analyses and interpretation of the data were performed by Syim Salahuddin, Dr. Cecilia Costiniuk, Dr. Mohammad-Ali Jenabian and Dr. Nicolas Chomont.

3.1 Characteristics of study population

24 HIV^+ adults were enrolled in this study, with demographic and clinical parameters included in Table 2. Seven participants were current tobacco smokers. Median age was 52 (IQR: 47-58) years, duration of HIV infection was 15 (IQR: 12-25) years, and duration of viral load suppression was 9 (IQR: 4-10) years. A minimum of 3 years of HIV suppression was selected since the number of HIV-infected cells, as determined by HIV DNA levels in CD4 T cells, typically declines during the initial 1 to 3 years of ART, then reaches a stable level that does not decline further during subsequent treatment (313). The number of study participants on integrase inhibitors was 16 (67%), while six (25%) were on protease inhibitor-based regimens and four (17%) were on non-nucleoside reverse transcriptase inhibitor-based regimens. Median peripheral blood CD4 T cell count was 558 (IQR: 430-876) cells/mm^3, CD4% was 32 (IQR: 27-37) and CD4/CD8 ratio was 0.7 (IQR: 0.60-0.97). In addition, we also recruited 8 HIV-uninfected participants without any respiratory symptoms based on their spirometric evaluation. Median age was 61 (IQR: 56-64) years, median peripheral blood CD4 T cell count was 531 (IQR: 360-572) cells/mm^3, CD4% was 41 (IQR: 37-48) and CD4/CD8 ratio was 2.25 (1.93, 2.68). Table 2 summarizes the demographic, clinical and lifestyle information of the study populations.

	ART-treated HIV$^+$ adults N=24	Healthy adults N=8
Demographic factors		
Age in years, median (IQR)	52 (47,58)	61 (56, 64)
Male sex, n (%)	19 (79%)	8 (100%)
Ethnicity, n (%)		
Caucasian	17 (71%)	8 (0%)
Black/Caribbean	3 (13%)	0 (0%)
Black/African	2 (8%)	0 (0%)
Hispanic	2 (8%)	0 (0%)
HIV-related factors		
Duration of HIV infection in years, median (IQR)	15 (12, 25)	N/A
Duration since viral load suppressed in years, median (IQR)	9 (4, 10)	N/A
Antiretroviral regimen, n (%)*		
Integrase inhibitor	16 (67%)	N/A
Non-nucleoside reverse transcriptase inhibitor	4 (17%)	N/A
Protease inhibitor	6 (25%)	N/A
CD4 count in cells/mm^3, median (IQR)	558 (430, 876)	531 (360, 572)
CD4 percentage, median (IQR)	32 (27, 37)	41 (37, 48)
CD4/CD8 ratio	0.7 (0.6, 0.97)	2.25 (1.93, 2.68)
Lifestyle factors		
Tobacco smoker, n (%)		
Current	7 (29%)	1 (13%)
Ever	12 (50%)	2 (25%)
Never	5 (21%)	5 (63%)

Table 2: Demographic, clinical and lifestyle parameters of both study groups. IQR: interquartile range. *Note: 1 participant was on a regimen containing both an integrase inhibitor and a protease inhibitor; 1 participant was on regimen containing both an integrase inhibitor and a non-nucleoside reverse transcriptase inhibitor.

3.2 HIV persistence in the lungs of ART-treated HIV-infected individuals

Ultrasensitive RT-PCR was performed to quantify the frequency of cells harboring HIV DNA in matched BAL cells, lung biopsies (approximately 2 to 3 mm deep) and PBMCs. The mean number of total HIV DNA copies per million cells was significantly higher in BAL cells compared to PBMCs and to biopsies (3910±10717 copies/10^6 cells *vs* 296.9±307.1 copies/10^6 cells, p=0.009; and 47.00±42.52 copies/10^6 cells, p=0.001, Figure 7A), suggesting that the surface of the mucosa was enriched in HIV-infected cells when compared to blood and pulmonary tissues. Frequencies of cells harboring HIV DNA in BAL cells and PBMCs were positively correlated (Figure 7B). Total HIV DNA was then quantified in FACS-sorted CD4 T cells from the BAL fluid, isolated CD4 T cells from peripheral blood and FACS-sorted alveolar macrophages. CD4 T cells from the BAL fluid harbored higher levels of total HIV DNA compared to peripheral CD4 T cells and alveolar macrophages, though significance could not be reached for the latter cell subset (Figure 7C). Importantly, in a subset of study participants in whom we had sufficient number of BAL cells (n=7), cell-associated HIV RNA was detected in BAL from 4 participants (Figure 7D). Although the levels of HIV RNA did not differ between BAL cells and PBMCs, this suggested that pulmonary mucosal cells may serve as a site of active viral replication under suppressive ART. Altogether, these observations demonstrate clearly that the lungs are enriched in CD4 T cells harboring high levels of HIV DNA in individuals despite long-term ART.

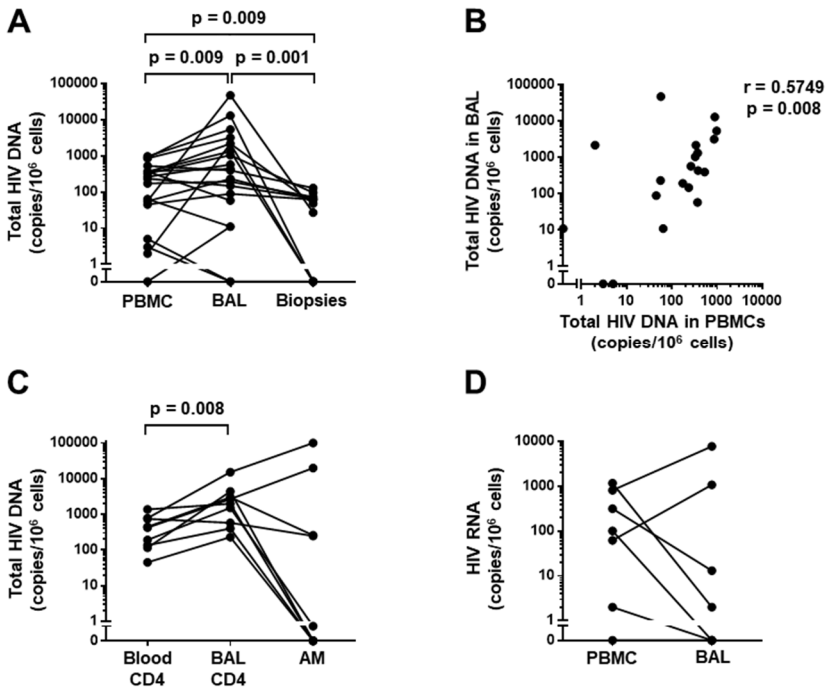

Figure 7: Quantification of total HIV DNA and cell-associated HIV RNA. (A) Total HIV DNA in PBMCs, BAL cells and pulmonary biopsies (copies/10^6 cells). (B) Correlation analysis of HIV DNA in BAL cells *vs* PBMCs. (C) Total HIV DNA in CD4 T cells purified from PBMCs, and CD4 T cells and alveolar macrophages purified from BAL cells (copies/10^6 cells). (D) Cell-associated HIV RNA in BAL cells *vs* PBMCs. Of note, statistical analyses are mentioned within the figures only if a significant difference is detected. “Alveolar macrophages” is abbreviated “AM”.

3.3 Differentiation phenotype of pulmonary mucosal CD4 T cells compared to blood

T cell subsets among BAL cells *vs* PBMCs were phenotypically characterized as described in Figure 8A. The frequency of naïve CD4 T cells was significantly lower in the BAL fluid *vs* blood in both study arms (HIV^+: 0.954±1.31% *vs* 32.4±15.4%, p<0.0001; HIV^-: 4.08±9.8% *vs* 32.0±16.8%, p=0.008; Figure 8B). The frequency of TD CD4 T cells was also lower in the BAL fluid *vs* blood in both study arms (HIV^+: 0.65±0.61% *vs* 5.10±3.86%, p<0.0001; HIV^-: 0.786±0.906% *vs* 9.46±14.2%, p=0.02; Figure 8E). Conversely, EM CD4 T cells, a known cellular reservoir for intact HIV genomes (1, 314-316), were present at significantly higher frequencies among BAL cells compared to PBMCs in both HIV^+ and HIV^- participants (HIV^+: 52.7±22.0% *vs* 6.79±11.3%, p<0.0001; HIV^-: 55.3±12.7% *vs* 1.14±1.62%, p=0.008; Figure 8D). Frequencies of CM CD4 T cells did not differ between the pulmonary and peripheral compartments in HIV-infected individuals but did differ significantly in healthy volunteers (HIV^-: 39.5±13.1% *vs* 57.2±13.7%, p=0.03; Figure 8C).

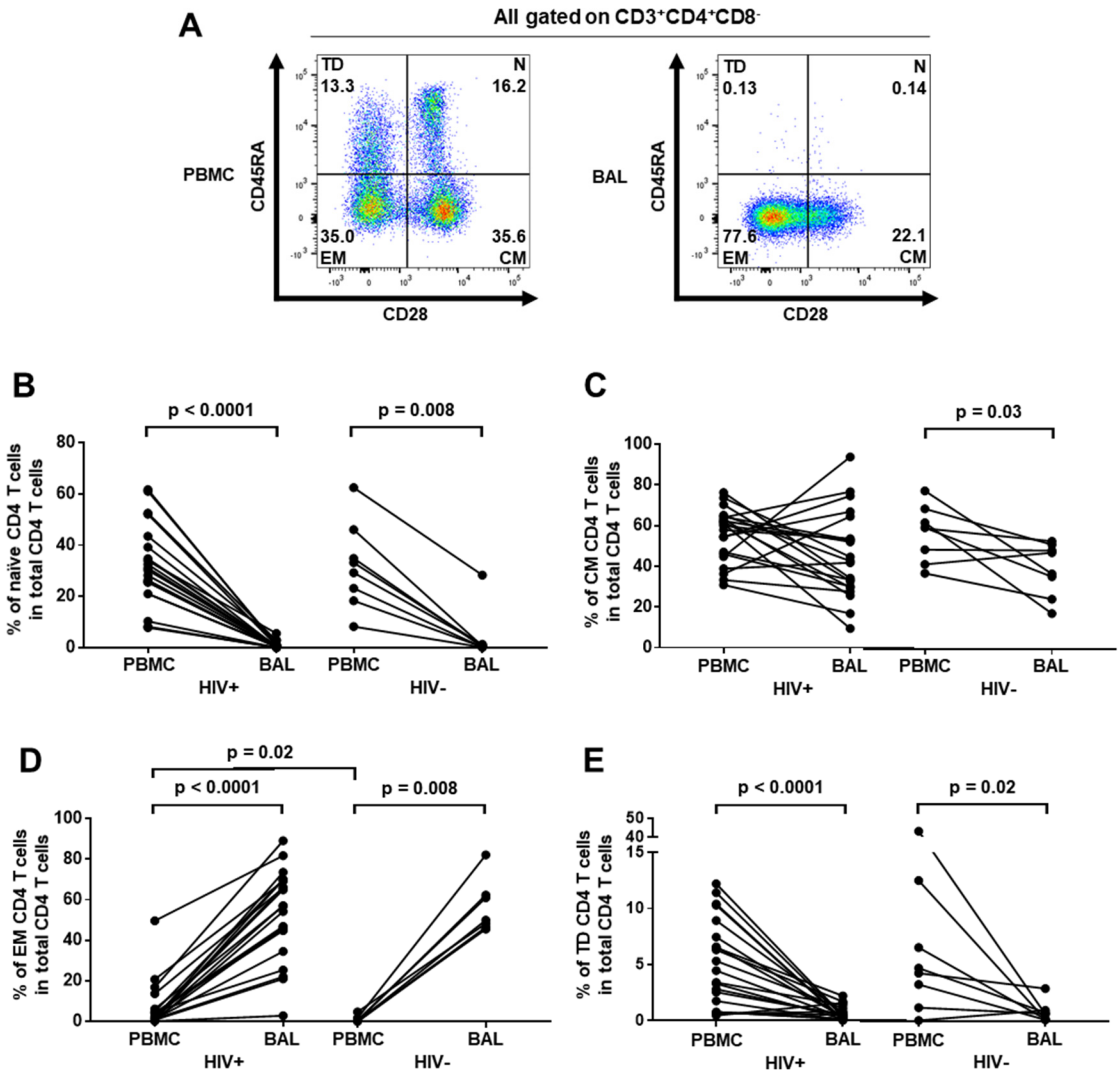

Figure 8: Differentiation phenotype of CD4 T cells in ART-treated HIV-infected participants and healthy participants. (A) Gating strategy used in flow cytometry to define CD4 T cell subsets. Naïve (N), central memory (CM), effector memory (EM), and terminally differentiated (TD) CD4 T cells were defined as $CD45RA^{+}CD28^{+}$, $CD45RA^{-}CD28^{+}$, $CD45RA^{-}CD28^{-}$, and $CD45RA^{+}CD28^{-}$, respectively. Percentages of (B) naïve, (C) central memory, (D) effector memory and (E) terminally differentiated CD4 T cells among total CD4 T cells in BAL compared to blood. Of note, statistical analyses are mentioned within the figures only if a significant difference is detected.

3.4 Immune activation and immune senescence of CD4 T cells

T cell subsets among BAL cells *vs* PBMCs were phenotypically characterized as described in Figure 9A to assess levels of immune activation and immune senescence. Higher frequencies of CD4 T cells expressing the immune activation marker HLA-DR was observed in the BAL fluid of both groups compared to blood (HIV^+: 27.0±12.2% *vs* 5.22±2.70%, p<0.0001; HIV^-: 32.5±25.9% *vs* 5.00±4.90%, p=0.008; Figure 9B). When looking at co-expression of both activation markers HLA-DR and CD38, only the HIV-infected group showed a significant increase in activated CD4 T cells in the lungs compared to blood (HIV^+: 3.68±4.24% *vs* 1.38±0.729%, p=0.002; Figure 9C). Interestingly, BAL cells of HIV-infected individuals expressed higher levels of $HLA\text{-}DR^+CD38^+$ CD4 T cells compared to BAL cells of HIV-uninfected individuals (p=0.04; Figure 9C). Meanwhile, BAL CD4 T cells were more senescent ($CD57^+CD28^-$) compared to their circulating counterparts in both groups (HIV^+: 22.4±14.8% *vs* 6.15±14.3%, p=0.002; HIV^-: 16.6±13.3% *vs* 0.432±0.367%, p=0.02; Figure 9D). Altogether, these results point to an important enrichment in the proportion of highly differentiated, activated and senescent CD4 T cells in the lungs when compared to blood in participants receiving long-term suppressive ART and in healthy volunteers.

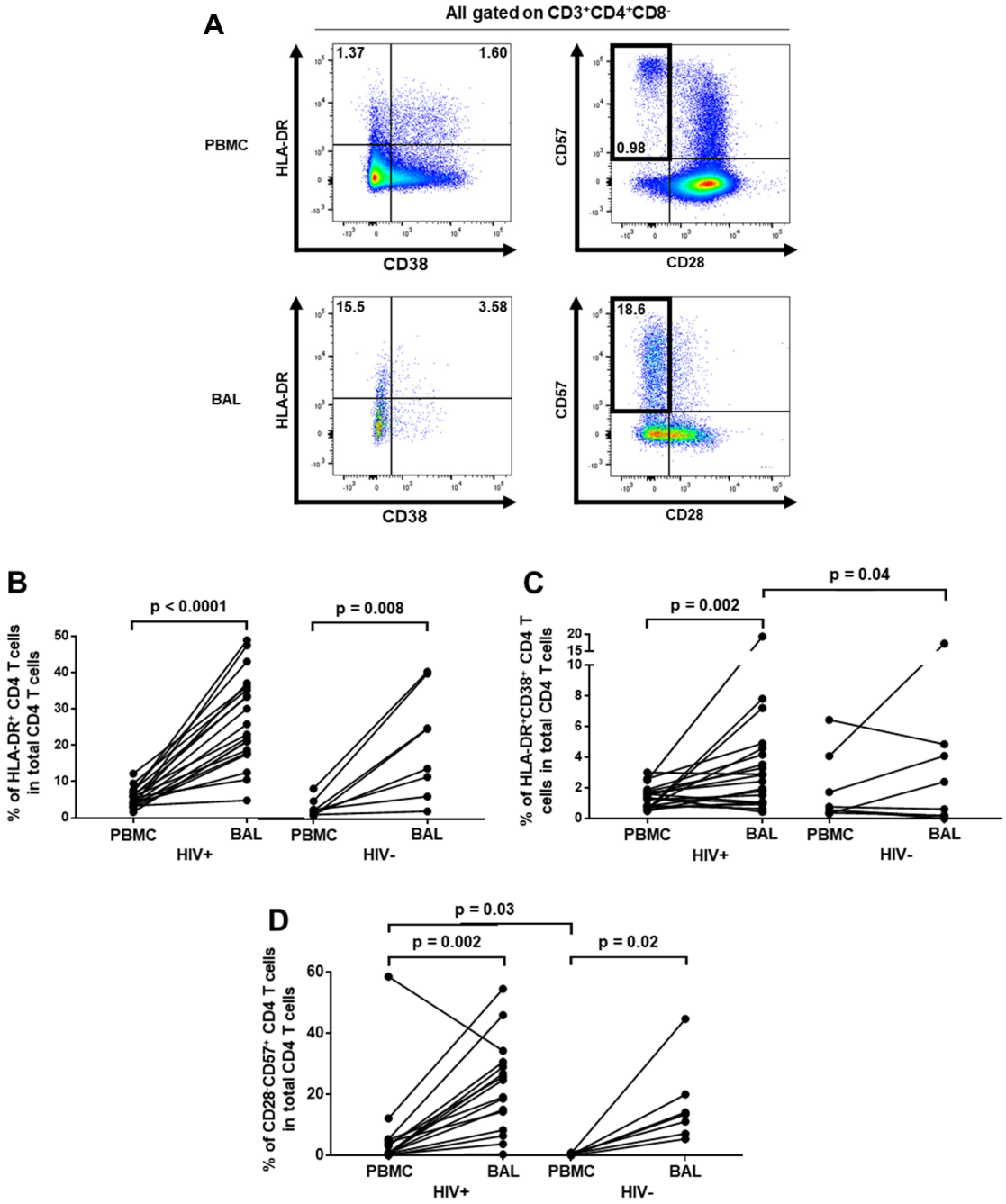

Figure 9: Immune activation and immune senescence of CD4 T cells. (A) Activated CD4 T cells were characterized as HLA-DR$^+$ and HLA-DR$^+$CD38$^+$, while senescent CD4 T cells were characterized as CD57$^+$CD28$^-$. (B) Percentage of activated HLA-DR$^+$ CD4 T cells and activated HLA-DR$^+$CD38$^+$ CD4 T cells among total CD4 T cells in BAL compared to blood. (D) Percentage of senescent CD4 T cells among total CD4 T cells in BAL compared to blood. Of note, statistical analyses are mentioned within the figures only if a significant difference is detected.

3.5 Distribution of memory CCR6$^+$ CD4 T cell subsets

We also assessed the presence of various CD4 T cell subsets previously described as preferential cellular reservoirs of HIV (170, 172, 173). The frequencies of memory CCR6$^+$, Th1Th17 (defined as CD45RA$^-$CCR6$^+$CCR4$^-$CXCR3$^+$) and memory CCR6$^+$ lacking the expression of CCR4 and CXCR3 (Figure 10A) within CD4 T cells were assessed. Memory CCR6$^+$ and Th1Th17 CD4 T cells were significantly higher in the BAL fluid *vs* blood of HIV-infected patients (HIV$^+$: 47.1±25.3% *vs* 28.2±17.5%, p=0.002; 14.6±14.9% *vs* 3.83±2.70%, p=0.002; Figure 10B, C). However, in healthy participants, only memory CCR6$^+$ CD4 T cells were enriched in the BAL fluid *vs* blood (HIV$^-$: 45.1±17.6% *vs* 26.9±7.04%, p=0.009). Moreover, the memory CCR6$^+$CCR4$^-$CXCR3$^-$ CD4 T cell subset, another recently proposed HIV reservoir (173), was also enriched in the BAL fluid *vs* blood (HIV$^+$: 11.4±11.4% *vs* 6.08±8.75%, p=0.008; HIV$^-$: 9.83±5.30% *vs* 3.92±3.35%, p=0.03; Figure 10D).

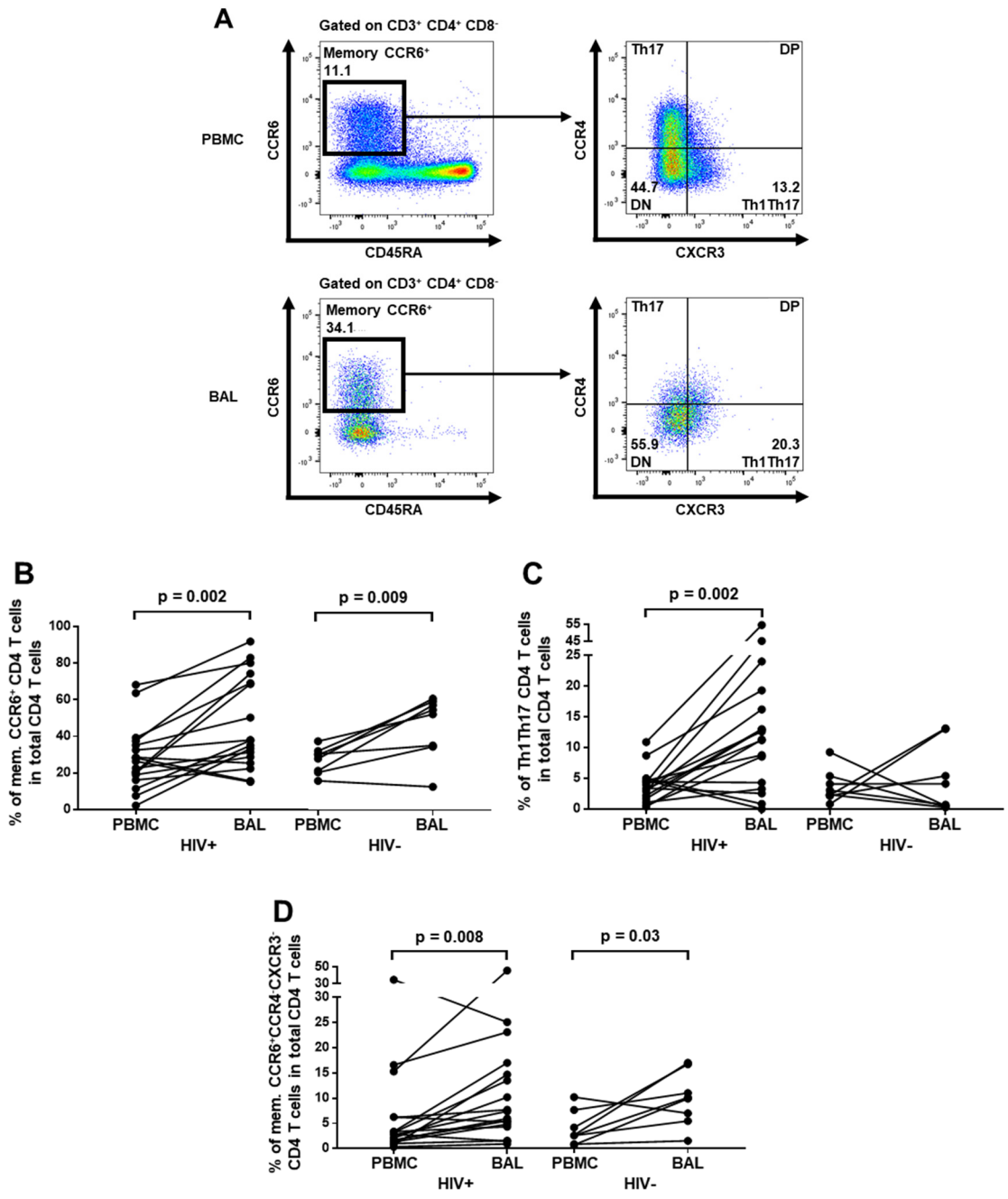

Figure 10: Distribution of memory CCR6$^+$ CD4 T cell subsets. (A) Gating strategy used in flow cytometry to isolate memory CCR6$^+$, Th1Th17 and memory CCR6$^+$CCR4$^-$CXCR3$^-$ CD4 T cells. Percentage of (B) memory CCR6$^+$, (C) Th1Th17 and (D) memory CCR6$^+$CCR4$^-$CXCR3$^-$ CD4 T cells among total CD4 T cells in BAL compared to blood. Of note, statistical analyses are mentioned within the figures only if a significant difference is detected.

3.6 Frequencies of CD39^{+} regulatory CD4 T cells

In other immunological effector tissues such as the gut mucosa, higher frequencies of immunosuppressive total Tregs and CD39^{+} Tregs as well as the imbalance between Tregs and effector T cells were described during chronic HIV infection (317). Our team also showed previously that these cells are involved in HIV disease progression by inhibiting anti-HIV specific responses (318). CD39 is an ectonucleotidase which hydrolyzes inflammatory adenosine triphosphate (ATP) into adenosine monophosphate (AMP), linking Tregs to the mechanism of ATP breakdown and potentially to the production of immunosuppressive adenosine. Within Tregs defined as CD127^{low}CD25^{+}FoxP3^{+}, we observed a higher proportion of Tregs expressing CD39 in the BAL fluid compared to blood of HIV-infected participants (HIV^{+}: 62.6±19.3% *vs* 48.7±18.9%, p=0.008; Figure 11B), suggesting their potential role in the pulmonary compartment in reducing anti-HIV responses.

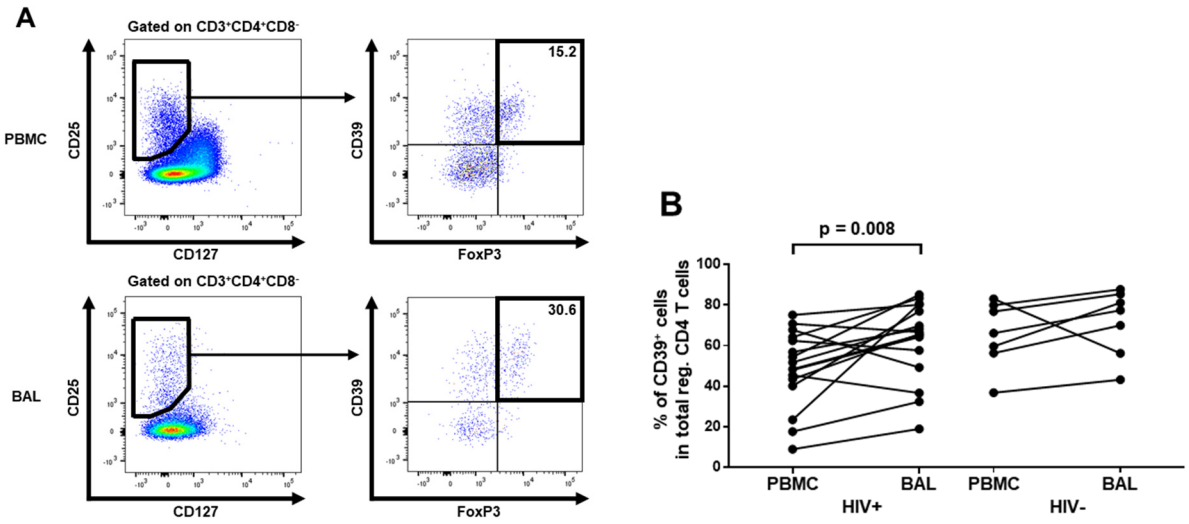

Figure 11: Enrichment in CD39^{+} regulatory T cells in the lungs of ART-treated HIV-infected adults. (A) Regulatory CD4 T cells were characterized as CD127^{low}CD25^{+}FoxP3^{+}. (B) Percentage of CD39^{+} Tregs among total regulatory CD4 T cells in BAL compared to blood. Of note, statistical analyses are mentioned within the figures only if a significant difference is detected.

3.7 Phenotypic characterization of double negative T cells

Another proposed cellular HIV reservoir are $CD3^+CD4^-CD8^-$ DN T cells, which may be generated as a consequence of the downregulation of CD4 by HIV accessory proteins Nef or Vpu during productive infection (197, 319, 320). Here, DN T cells were defined in two manners, as $CD3^+CD4^-CD8\alpha\alpha^-CD8\alpha\beta^-$ or $CD4^-CD8^-TCR\alpha\beta^-TCR\gamma\delta^-$ (Figure 12A). Under both definitions, the frequencies of DN Tcells were significantly higher in the BAL fluid compared to blood in ART-treated HIV^+ individuals (HIV^+: 16.1±15.5% *vs* 4.98±2.53%, p=0.0002; 21.1±17.9% *vs* 10.5±22.8%, p=0.005, respectively; Figures 12B, C), an increase not seen in healthy individuals which may indicate that downregulation of CD4 was achieved by HIV. In addition, in HIV^+ individuals, BAL *vs* PBMC DN T cells exhibited higher levels of immune activation (HIV^+: 14.9±9.24% *vs* 8.58±4.35%, p=0.01; Figure 12D) and lower levels of the immune senescence (HIV^+: 20.0±10.7% *vs* 29.9±16.0%, p=0.003; Figure 12E), which is not the case in healthy individuals. To determine if these DN T cells are recent thymic emigrants (RTE), the expression of the RTE marker CD31 (321) was measured. DN T cells expressed lower levels of CD31 in the BAL fluid *vs* blood (HIV^+: 35.9±15.9% *vs* 54.6±13.2%, p=0.002; Figure 12F) in HIV^+ individuals, suggesting that these DN T cells present in the BAL fluid were not recently generated from the thymus, while no significant difference was observed in healthy participants. Interestingly, DN T cells recently migrated from the thymus were observed at a higher frequency in the lungs of healthy volunteers compared to the lungs of HIV-infected patients (p=0.02; Figure 12F). These results demonstrated the abundant presence of DN T cells in the BAL fluid of ART-treated HIV-infected adults and suggest that these cells likely represent a more advanced stage of T cell differentiation.

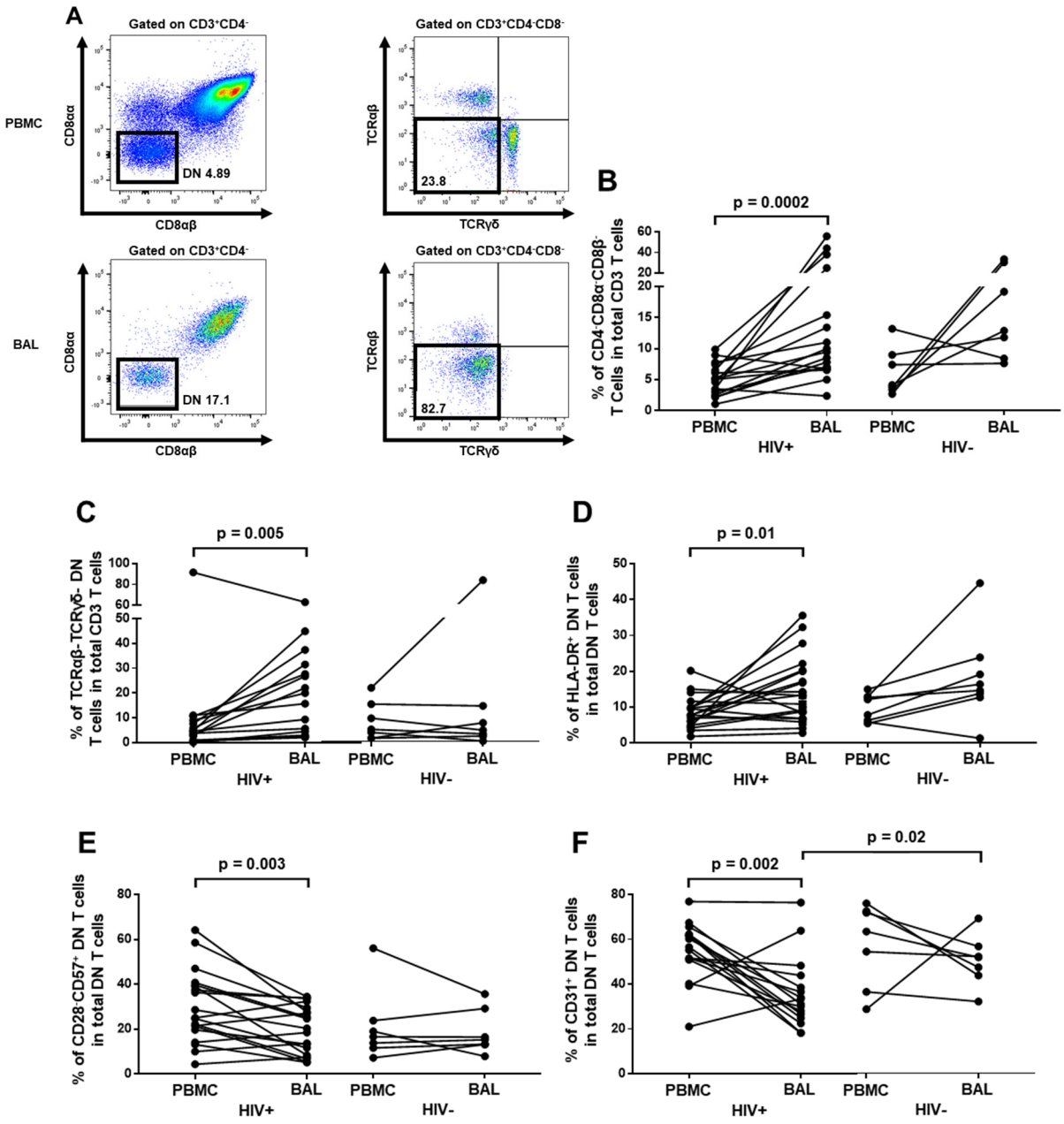

Figure 12: Distribution and phenotypic characterization of DN T cells. (A) Two strategies of gating used in flow cytometry for DN T cell identification based respectively on $CD4^-CD8\alpha\alpha^-CD8\alpha\beta^-$ and $CD4^-CD8^-TCR\alpha\beta^-TCR\gamma\delta^-$ phenotypes. Percentage of DN T cells defined as (B) $CD4^-CD8\alpha\alpha^-CD8\alpha\beta^-$ and (C) $CD4^-CD8^-TCR\alpha\beta^-TCR\gamma\delta^-$ among total CD3 T cells in BAL compared to blood. Percentage of (D) activated and (E) senescent DN T cells among total DN T cells in BAL compared to blood. (F) Percentage of DN T cells recently migrated from the thymus in BAL compared to blood. Of note, statistical analyses are mentioned within the figures only if a significant difference is detected.

3.8 Expression of CD32a on pulmonary mucosal CD4 T cells of ART-treated adults

CD32a is the low affinity Fcγ type II receptor recently proposed as a marker of quiescent CD4 T cells enriched in HIV cellular reservoirs in the blood of virally suppressed individuals (198). To determine the potential role of $CD32a^+$ T cells in HIV persistence during ART in the lungs, the expression of CD32a was quantified on CD4 T cell subsets from the BAL fluid *vs* blood. To properly assess CD32a expression, an isotype control IgG2b was used to eliminate nonspecific binding signals of antibodies (Figure 13A). The frequency of $CD32a^+$ cells was significantly higher in CD4 T cells from BAL cells compared to PBMCs (HIV^+: 1.89±2.31% *vs* 0.168±0.173%, p=0.005; Figure 13A) of HIV-infected individuals under long-term suppressive ART. Interestingly, higher levels of pulmonary mucosal CD4 T cells co-expressing CD32a and the HIV co-receptor CCR5 were observed in the BAL fluid compared to blood (HIV^+: 0.413±0.493% *vs* 0.0542±0.0798%, p=0.04; Figure 12B). In addition, CD4 T cells co-expressing CD32a and the activation marker HLA-DR were found in greater frequency in the BAL fluid *vs* blood (HIV^+: 0.296±0.452% *vs* 0.0407±0.0281%, p=0.02; Figure 13C). 92.5±9.08% of pulmonary $CD32a^+$ CD4 T cells were $HLA\text{-}DR^+$ compared to 37.9±34.3% in the blood. These results demonstrate an enrichment of HIV permissive and activated $CD32a^+$ CD4 T cells in the lung mucosa.

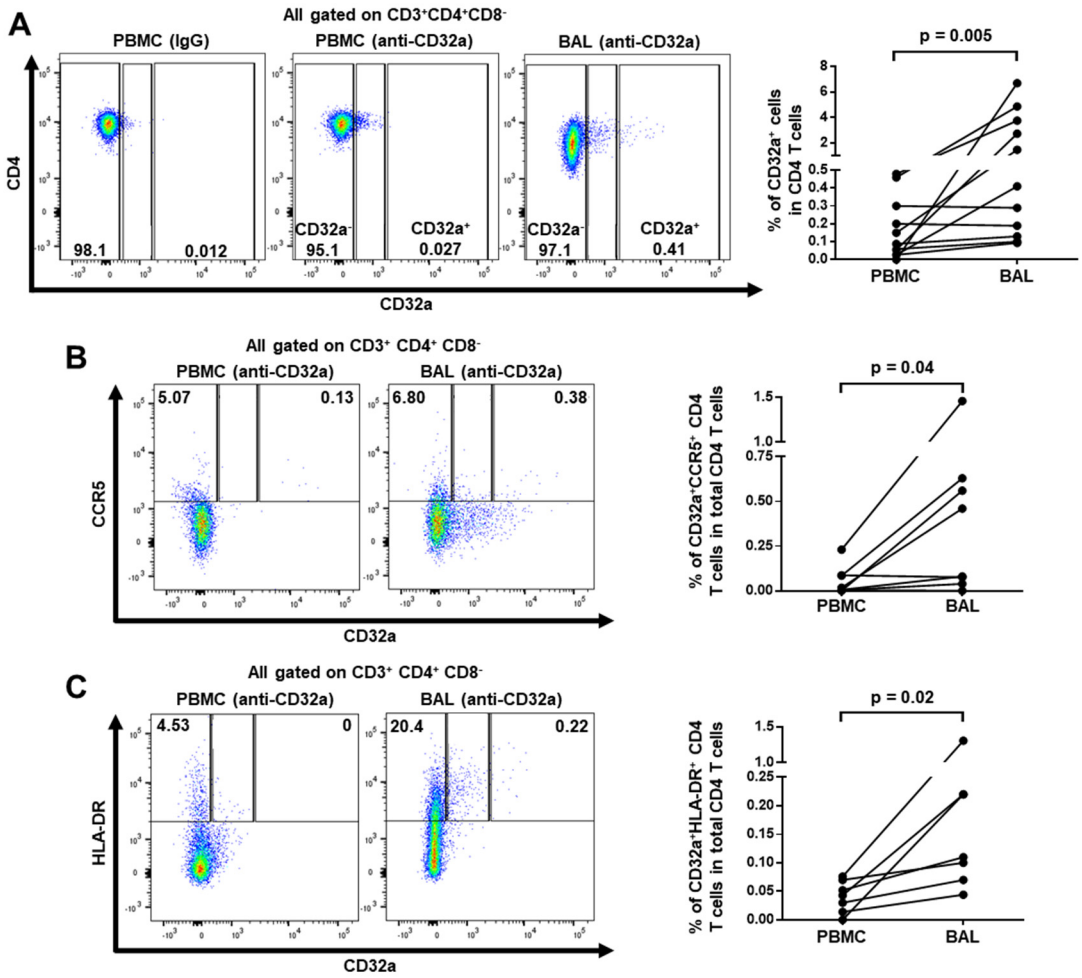

Figure 13: Frequency and characterization of CD4 T cells expressing CD32a. (A) Gating strategy used in flow cytometry to quantify CD32a$^+$ T cells using IgG2b κ isotype controls. Percentage of CD32a$^+$ cells among total CD4 T cells in BAL compared to blood. (B) Representative figure showing the co-expression of CD32a and CCR5 on pulmonary mucosal CD4 T cells, and percentage of CD4 T cells co-expressing CD32a and CCR5 among total CD4 T cells in BAL compared to blood. (C) Representative figure showing the co-expression of CD32a and HLA-DR on pulmonary mucosal CD4 T cells, and percentage of CD4 T cells co-expressing CD32a and HLA-DR among total CD4 T cells in BAL compared to blood.

3.9 Differentiation phenotype of pulmonary mucosal CD8 T cells compared to blood

The frequency of naïve CD8 T cells was significantly lower in BAL cells *vs* PBMCs in both study arms (HIV^+: 4.44±7.16% *vs* 26.6±16.5%, p<0.0001; HIV^-: 4.92±8.72% *vs* 28.4±15.2%, p=0.001; Figure 14A). The frequency of TD CD8 T cells was also lower in the BAL fluid *vs* blood in both study arms (HIV^+: 10.6±9.52% *vs* 39.3±19.8%, p<0.0001; HIV^-: 7.13±8.00% *vs* 22.1±13.9%, p=0.001; Figure 14D). Among the memory CD8 T cell subsets, EM CD8 T cells were present at significantly higher frequencies among BAL cells compared to PBMCs in both HIV^+ and HIV^- participants (HIV^+: 66.6±23.4% *vs* 8.64±5.42%, p<0.0001; HIV^-: 61.8±18.4% *vs* 6.28±5.36%, p=0.008; Figure 14C), while frequencies of CM CD8 T cells decreased in the pulmonary *vs* peripheral compartments in HIV-infected individuals only (HIV^+: 16.8±14.7% *vs* 24.4±13.5%, p=0.04; Figure 14B).

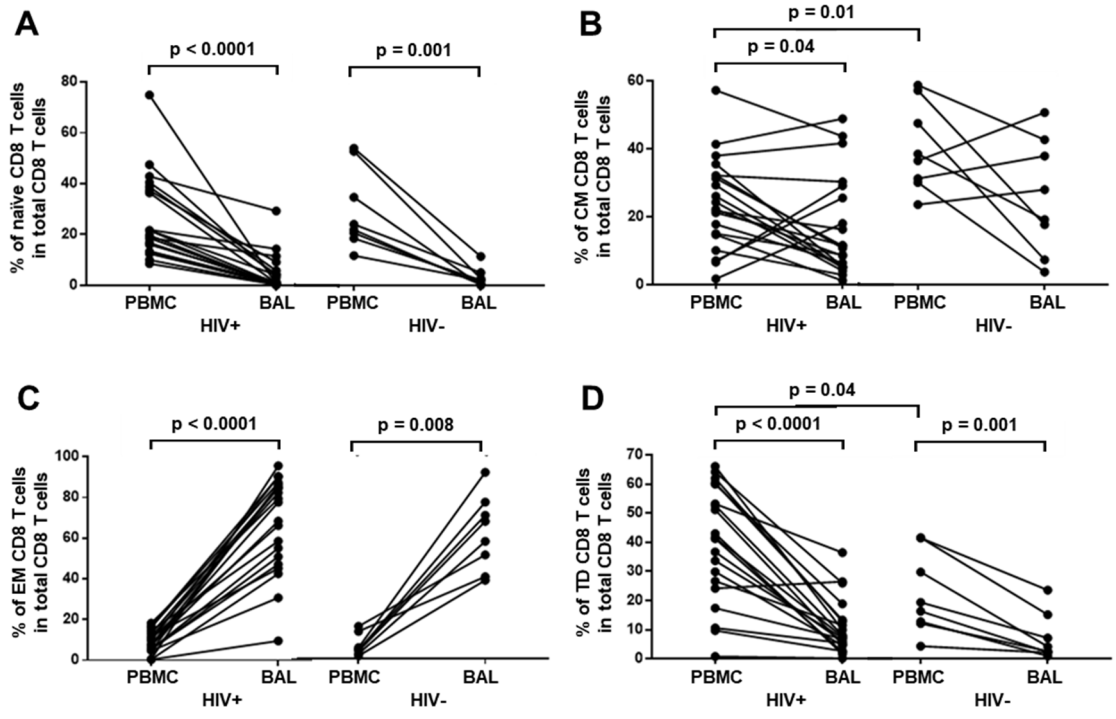

Figure 14: Differentiation phenotype of CD8 T cells in ART-treated HIV-infected participants and healthy participants. Percentages of (A) naïve, (B) central memory, (C) effector memory and (D) terminally differentiated CD8 T cells among total CD8 T cells in BAL compared to blood. Of note, statistical analyses are mentioned within the figures only if a significant difference is detected.

3.10 Immune activation, immune exhaustion and functional phenotype of CD8 T cells

Various phenotypic and functional characteristics of CD8 T cells were assessed, including levels of PD-1, Granzyme B and perforin (Figure 15A). Higher frequencies of CD8 T cells expressing the immune activation marker HLA-DR was observed in the BAL of both groups compared to blood (HIV^+: 15.8±10.5% *vs* 5.26±4.36%, p<0.0001; HIV^-: 30.0±20.6% *vs* 8.13±5.80%, p=0.02; Figure 15B). As discussed previously, generalized cellular immune activation in CD8 T cells have been linked to accelerated exhaustion and immune dysfunction. As such, PD-1 was assessed in CD8 T cells of HIV-infected individuals. Pulmonary mucosal CD8 T cells expressed higher levels of PD-1 than their circulating counterparts in ART-treated patients (HIV^+: 37.3±24.5% *vs* 6.74±3.34%, p=0.01; Figure 15C). It was previously demonstrated that CD8 T cells expressing CD73, an ectonucleotidase involved in the generation of immunosuppressive adenosine, are antigen-specific cells in the context of HIV infection and HIV elite controllers are characterized with elevated frequencies of these cells (322). Importantly, lower frequencies of $CD73^+$ CD8 T cells were observed in the BAL fluid compared to blood in both study arms (HIV^+: 10.8±9.42% *vs* 30.7±16.6%, p=0.0004; HIV^-: 11.2±4.52% *vs* 35.6±15.8%, p=0.01; Figure 15D). We then assessed the intracellular expression of Granzyme B and perforin on CD8 T cells as Granzyme B is involved in the induction of apoptosis, while perforin generates pores on target cells to allow the entry of Granzyme B (323). Notably, a massive decrease in perforin expression and lower levels of Granzyme B were observed in pulmonary mucosal CD8 T cells *vs* blood CD8 T cells (HIV^+: 5.04±11.1% *vs* 20.7±21.1%, p=0.008 and 35.8±14.0% *vs* 46.5±13.4, p=0.12, respectively; Figure 15E). Altogether, these observations suggest that pulmonary CD8 T cells may have less anti-HIV and cytotoxic functions, which may in turn contribute in HIV persistence within the lungs despite suppressive ART.

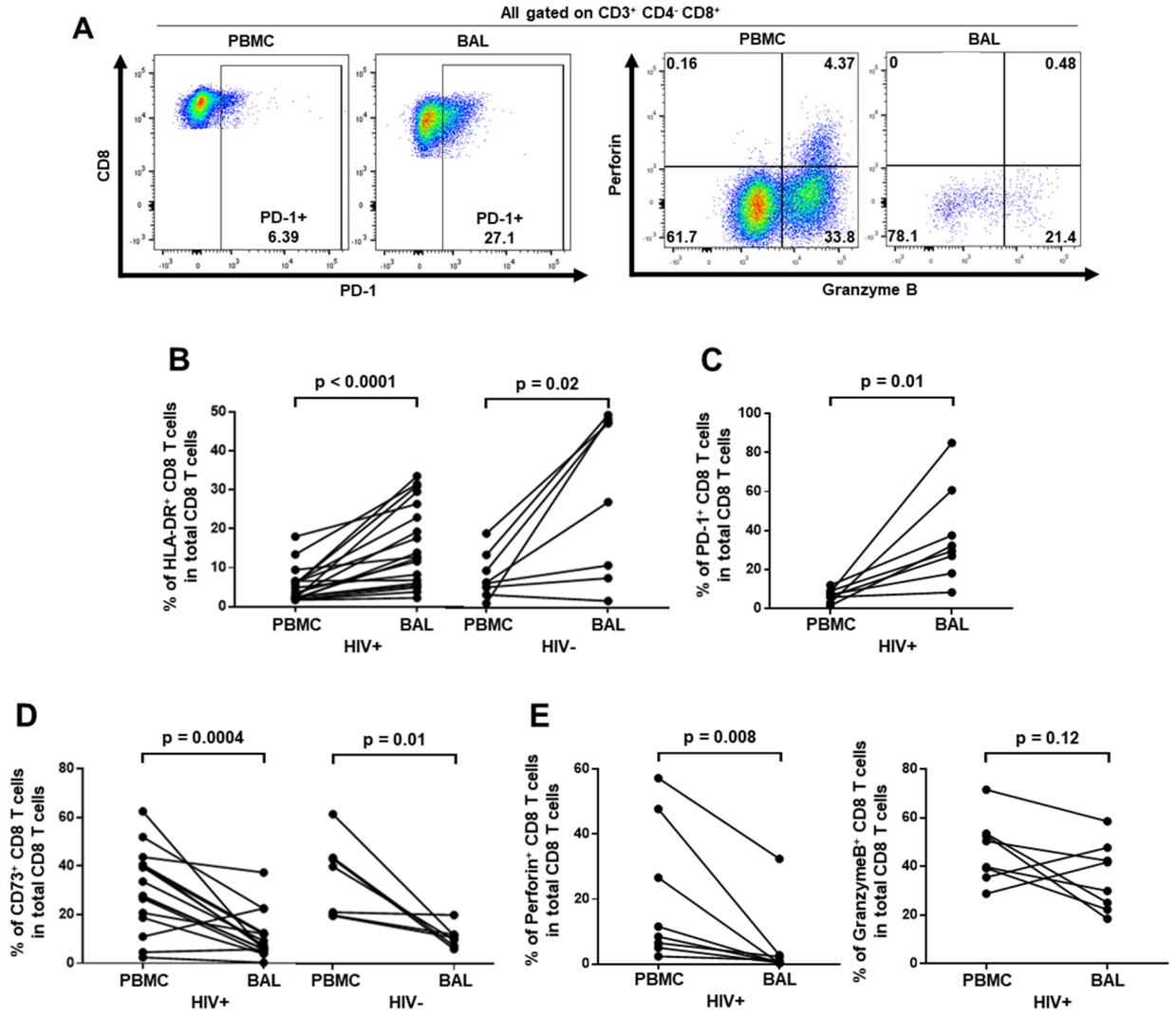

Figure 15: Functional phenotype of CD8 T cells. (A) Gating strategy used in flow cytometry to identify exhausted and dysfunctional cells. Percentages of (B) activated and (C) exhausted CD8 T cells, (D) percentage of CD8 T cells expressing CD73, and (E) percentages of CD8 T cells expressing perforin and Granzyme B among total CD8 T cells in BAL compared to blood. Of note, statistical analyses are mentioned within the figures only if a significant difference is detected. Panel E is an exception wherein the p-value for the comparison of Granzyme B expression is shown despite not reaching significance, perhaps due to the small sample size.

CHAPTER 4: DISCUSSION

Author contribution: The following chapter was written by Syim Salahuddin, with suggestions from Dr. Cecilia Costiniuk and Dr. Mohammad-Ali Jenabian.

In this study, we provided evidence that the lungs contribute to HIV persistence in individuals receiving long-term suppressive ART. We performed an extensive phenotypic characterization of pulmonary mucosal CD4 T cell subsets. Previous studies performed before the introduction of ART revealed the role of alveolar macrophages in HIV replication (2). This is consistent with the fact that opportunistic infections targeting the pulmonary compartment and increased risk of TB are associated with HIV infection (2, 238, 267, 303). However, the role of pulmonary mucosal CD4 T cells in HIV persistence within the lungs remained unclear. Our study is one of the few that addresses the important question of HIV persistence within the lungs in the era of ART. Although one cannot exclude the contribution of alveolar macrophages to HIV persistence during ART (277, 301), it is globally accepted that memory CD4 T cells constitute the major cellular reservoir of HIV wherein the virus remains in a latent form (1). In line with this idea, our study reveals the significant infiltration in the pulmonary mucosa of various CD4 T cell subsets previously identified in the blood as important reservoirs of HIV. These findings in turn underscore the necessity to consider the pulmonary HIV burden in therapeutic strategies aimed at eradicating the virus.

We found that the mean total HIV DNA copies/10^6 cells was 13-fold higher in total BAL cells *vs* PBMCs of participants on long-term suppressive ART (median time of viral suppression: 9 years). Importantly, the levels of HIV DNA were correlated between both pulmonary and peripheral compartments. Interestingly, we found higher HIV DNA levels in total BAL cells *vs* pulmonary biopsies, suggesting that viral DNA is seeded mainly within cells at the mucosal levels. As alveolar macrophages were reported to be infected by HIV (277), we assessed whether they or CD4 T cells are enriched in HIV DNA by FACS-sorting matched pulmonary mucosal CD4 T cells, alveolar macrophages and peripheral CD4 T cells. We found that CD4 T cells in the lung mucosa represent the highest HIV burden within the lungs as these cells contain greater HIV DNA levels compared to alveolar macrophages, except in two study participants. The measurement of total HIV DNA is known to overestimate the size of the replication-competent HIV reservoir given that most viral DNA is defective (324). Although it will be important to confirm our observation by using functional assays, several studies have found that total HIV DNA can be used as a surrogate

to determine the frequency of cells capable of producing viral proteins (316) and even infectious viruses (325). Importantly, we were able to detect cell-associated HIV RNA in BAL cells from 4 out of 7 participants, suggesting that these cells may support persistent levels of viral transcription during long-term suppressive ART. These findings are consistent with the fact that antiretroviral drugs do not act on viral transcription (326), thus allowing residual viral transcription to occur during ART. Such residual viral transcription occurs mainly at barrier surfaces where T cells are activated through interactions with the microbial flora. This was clearly reported for gut-associated lymphoid tissue by other groups (327) and by our current results within the lungs.

In both ART-treated HIV-infected patients and healthy individuals, we observed a significant reduction in the frequency of naïve CD4 T cells, and conversely, a dramatic enrichment in effector memory CD4 T cells in BAL cells *vs* PMBCs. The immune system is organized in primary and secondary lymphoid tissues, and peripheral effector sites including the lungs (328, 329). Therefore, the enrichment of effector cells is expected given that pulmonary mucosal tissues are immunological effector sites that are constantly exposed to infectious and environmental agents, and undergo chronic antigenic stimulation. Effector memory CD4 T cells were previously identified as the main subset supporting HIV transcription in both peripheral blood and intestinal tissues in ART-treated adults (330). Importantly, in peripheral blood of individuals on ART, effector memory CD4 T cells may harbor the majority of the intact and replication competent HIV DNA in the midst of other memory T cells (1, 315, 316, 325). They promote HIV persistence via their ability to undergo homeostatic proliferation which maintains and maybe increases the number of infected cells independently of *de novo* infection after prolonged ART (312).

Another important observation we made was the increased frequency of activated CD4 T cells at the mucosal surface of the lungs compared to blood. CD4 T cell activation is important as cellular immune activation may contribute to HIV persistence through both promotion of HIV replication and enhanced susceptibility of bystander cells to infection (331). Immune activation drives cycles of cellular expansion until these cells eventually reach a replicative limit, characterized by the loss of co-stimulatory receptor CD28 in senescent T cells which are in cell-

cycle arrest (332). Senescent T cells are dysfunctional and less capable of clearing infections. They also contribute to persistent upregulation of the pro-inflammatory response (332). Repeated exposures to tobacco, environmental toxins and infectious agents induce repeated stress on the lungs and may result in immune activation and immune senescence. The accumulation of senescent T cells could impair the cell maintenance and repair capabilities of the lungs and promote tissue destruction in the long-term (332). In HIV^+ adults, COPD and lung cancers are reported much earlier than in the general population (238). It must be underscored that the elevated risk of lung diseases and cancer in HIV infection is independent of smoking (238). Therefore, the high CD4 T cell differentiation, activation and senescence could contribute to allowing HIV to establish a viral reservoir in the lungs upon infection, and to heighten the HIV burden and mucosal pro-inflammatory milieu post-infection (238).

$CCR6^+$ Th17 cells, which are attracted to the gut and vaginal mucosae, are preferentially infected very early following infection and harbor high levels of replication-competent HIV DNA compared to $CCR6^-$ T cells despite suppressed viral load by ART (167, 170, 333). In addition, Th1Th17 cells express molecular signatures associated with HIV permissiveness (168) and contain high levels of HIV DNA under suppressive ART (171, 173). Furthermore, memory $CCR6^+CXCR3^-CCR4^-$ CD4 T cells, another subset of Th cells, have been proposed as another preferential reservoir for HIV (173). Of particular interest, we found higher frequencies of memory $CCR6^+$, Th1Th17 and memory $CCR6^+CXCR3^-CCR4^-$ CD4 T cells in the pulmonary mucosa *vs* blood, highlighting the potential contribution of various lymphoid subsets to higher HIV burden within the lungs. In line with our data, Brenchley *et al*. observed that in contrast to the gut, Th17 cells were not preferentially lost from the BAL fluid of HIV-infected individuals (334). Considering the limitations in performing HIV reservoir measurement on rare cell subsets from the lungs, whether lung-infiltrating Th17 cells comprise cellular HIV reservoirs in the lungs remains an open question.

Our lab and others have previously shown that higher levels of immunosuppressive Tregs and the imbalance of effector T cells and Tregs in the blood and gut mucosa is associated with

suppression of anti-HIV T cell responses (318, 335). Here, we observed a higher frequency of $CD39^+$ Tregs in the lungs *vs* blood of HIV-infected adults. $CD39^+$ Tregs are involved in HIV disease progression and suppression of anti-HIV T cell responses via the hydrolysis of inflammatory ATP into immunosuppressive adenosine in concert with another ectoenzyme, CD73 (318, 336). The increase in $CD39^+$ Tregs was not seen in healthy individuals. Therefore, higher $CD39^+$ Tregs in the lung mucosa of ART-treated patients could be HIV-induced. Indeed, it could be in response to highly activated effector cells in the pulmonary mucosa and/or of pro-inflammatory senescent T cells, phenomena observed in HIV^+ participants. Notwithstanding the cause for the emergence of $CD39^+$ Tregs, the latter could in the context of HIV infection be either beneficial, suppressing T cell activation, or harmful, decreasing HIV-specific T cell responses, cytokine production and T cell proliferation, thus contributing to viral persistence (318, 335).

We also examined $CD3^+CD4^-CD8^-$ DN T cells which could display both helper and immunoregulatory functions. The maintenance of high numbers of these cells which secrete Th cytokines is associated with immune activation (192). In primary HIV infection, DN T cells displayed immunosuppressive functions by IL-10 and TGF-β secretion to control immune activation (192). During chronic infection, a decline in DN T cells correlates with rapid disease progression (190). This immunosuppressive function could explain, at least in part, their higher frequencies within the lungs of HIV-infected individuals due to high levels of immune activation. Importantly, DN T cells have been identified as persistent HIV reservoirs that carry HIV Gag proteins despite ART (197). As opposed to healthy individuals, the lungs of HIV-infected individuals harbored higher frequencies of DN T cells compared to peripheral blood. These cells may therefore contribute significantly to HIV persistence within the lungs.

DN T cells may originate in the thymus by escaping negative selection followed by migration in the periphery where they expand upon experiencing antigens (337). To determine if pulmonary mucosal DN T cells originated from the thymus or developed extra-thymically, the recent thymic migration marker, CD31 (321), was measured. DN T cells expressed lower levels of CD31 in the BAL fluid *vs* blood in HIV-infected participants, suggesting that the DN T cells we

observed in the BAL fluid were not recent thymic emigrants. Also in the HIV^+ study arm, pulmonary DN T cells are less senescent compared to blood. A hypothesis is that these DN T cells had previously expressed the CD4 molecule but had lost CD4 expression following HIV infection. Indeed, it has been shown that Nef and Vpu proteins of HIV could downregulate CD4 from the cell surface of infected cells (319, 320). In line with this, frequencies of pulmonary DN T cells in healthy individuals did not differ compared to peripheral blood, which may lend support for the role of HIV in the emergence of DN T cells within the lungs. Furthermore, pulmonary DN T cells in healthy participants expressed CD31 in greater frequency compared to their counterparts in HIV-infected participants. This further supports the hypothesis that a subset of DN T cells in the lung mucosa of HIV-infected individuals did not escape the thymus recently and may have originated extra-thymically instead, perhaps in the lungs and as a result of HIV infection. CD4 downregulation can occur in infected T cells with minimal viral replication (197), and previous HIV infection of CD4 T cells could partially explain their enrichment within the lungs of ART-treated HIV-infected patients.

We also investigated the expression of the newly proposed marker of HIV latency in quiescent infected cells, CD32a, a low affinity immunoglobulin G Fc fragment receptor (198). We found higher expression of CD32a on pulmonary CD4 T cells *vs* their peripheral counterparts in HIV-infected individuals. Importantly, these cells expressed high levels of the activation marker HLA-DR within the lungs, which is in line with another recent study (199). Furthermore, following HIV infection of unstimulated PBMCs *ex vivo*, infected cells concomitantly upregulated the expression of both CD32 and HLA-DR (314). Indeed, productively infected cells expressing HIV RNA and Gag protein upregulated these markers by 2-folds (314). Although we assessed CD32a as a potential marker of HIV infection, it is not known whether $CD32^+$ pulmonary cells are indeed HIV-infected. Due to their very low proportion within pulmonary $CD4^+$ T-cells, we were not able to address this question by FACS-sorting.

Finally, we looked at the phenotypic and functional characteristics of CD8 T cells, which play a vital role in HIV-specific immune responses. In line with the effector aspect of the lungs as

an organ in constant contact with foreign organic and inorganic substances, there was an enrichment of effector memory CD8 T cells in both HIV-infected and healthy participants. Pulmonary mucosal CD8 T cells were also highly activated compared to their circulating counterparts. Importantly, both study groups showed a decrease in CD8 T cells expressing CD73, an immunosuppressive ectonucleotidase, in the lungs compared to blood. In the context of HIV, this observation is of particular importance as it was previously reported that $CD73^{+}$ CD8 T cells are antigen-specific cells and contribute in the processes of viral suppression in HIV elite controllers (322). We speculate that lower frequencies of HIV-specific pulmonary CD8 T cells may lead to weaker anti-HIV responses, in turn contributing to HIV persistence within the lungs, though HIV-specific functional tests will be required to test this hypothesis. In addition, pulmonary mucosal CD8 T cells in the HIV-infected study group demonstrated greater levels of immune exhaustion as seen by higher expression of PD-1, which may result in dysfunction and viremia (220) as well as contribute to viral persistence (221) as previously reported. Indeed, we observed an important decrease in perforin and Granzyme B (though statistical significance was not achieved for Granzyme B, likely due to the small sample size of assessed participants) in pulmonary CD8 T cells in HIV-infected adults. Indeed, pulmonary CD8 T cells are almost devoid of perforin and this may dramatically decrease their cytotoxic functions. Altogether, our observations suggest that potential dysfunctionality of pulmonary CD8 T cells could contribute to HIV persistence within the lungs despite long-term ART.

Our study has some limitations which merit discussion. The lungs are highly complex organs with various cells that synthesize an abundance of various fluids, antimicrobial proteins and mucins. It is unknown whether these cells may also be HIV reservoirs and how their products may contribute to HIV persistence. We attempted to examine FACS-sorted CD4 T cell subtypes known to be HIV reservoirs, but we were limited by cell numbers required for sorting since macrophages represent more than 90% of cells, while lymphocytes represent less than 10% of cells in the BAL fluid (250). In addition, at this point of the research project, we did not provide phenotypic analyses related to CD8 functions in uninfected individuals as we made the corresponding hypothesis only once we confirmed HIV persistence in FACS-sorted CD4 T cells. At

that point we were not able to recruit sufficient numbers of uninfected participants for these assessments. Moreover, anti-HIV and cytotoxic functional assays of pulmonary CD8 T cells need to be done to confirm our hypothesis. Another limitation of our study is that we did not perform genetic sequencing of HIV variants found in the BAL fluid *vs* peripheral blood cells. Studies from the early ART era showed much less sequence variability in HIV found within the lungs leading to complete phylogenetic separation of HIV lineages *vs* blood (308).

CONCLUSION AND SUMMARY

In summary, we provide evidence supporting the notion that the lungs are anatomical reservoirs of HIV during ART. In virally suppressed HIV^+ adults, the lungs' mucosal cells contain higher levels of HIV DNA compared to PBMCs and to lung biopsies. Cells that contribute to HIV reservoir persistence in the lungs include CD4 T cell types documented to carry HIV reservoirs in the blood, namely effector memory, memory $CCR6^+$, Th1Th17, memory $CCR6^+CCR4^-CXCR3^-$ and $CD32a^+$ CD4 T cells, as well as double negative T cells. In addition, there are a number of immune features and abnormalities observed in the pulmonary mucosal compartment compared to the peripheral blood, some pertaining to CD4 T cells such as the induction of immune senescence and of cells that may hinder anti-HIV responses, some pertaining to CD8 T cells such as cellular exhaustion, decrease in an HIV-specific cell subset and reduced cytotoxic potential, and some to both such as immune activation. In healthy individuals, effector memory and certain Th17 subsets known as HIV reservoirs were enriched. Upon infection, these can provide sanctuaries for HIV to establish latency. Immune activation and immune senescence of CD4 T cells remain, while CD8 T cells that can serve as HIV-specific cells are found in lower levels. This knowledge on the composition of immune cells infiltrating or residing within the lung mucosa is a necessary step towards elucidating the factors contributing to HIV persistence. While the advent of ART has greatly improved the quality of life of HIV-infected individuals and reduced AIDS-related mortality, the existence of cellular and anatomical reservoirs of HIV remains the primary barrier towards HIV eradication. Tremendous efforts brought about by scientists and clinicians from around the world geared towards identifying and characterizing latent HIV reservoirs have been crucial in improving our understanding of HIV/AIDS pathogenesis and improving clinical outcomes in HIV-infected individuals. Characterizing the pulmonary mucosal HIV reservoirs will bring us closer towards developing tissue-targeted therapeutic strategies and ultimately finding a cure to HIV. In conducting this study, we hope to contribute to the firmament of scientific, clinical, epidemiological, socioeconomic, societal and cultural works tirelessly put forth by people globally since the discovery of HIV/AIDS in order to help people infected with HIV lead a better, dignified and healthy life, and one day hopefully without HIV.

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