Effects of Smoking and HIV Infection on Lung Tissue-resident CD8 T-cell Dynamics in People Living with HIV on Antiretroviral Therapy

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ABSTRACT

Background: Despite the success of antiretroviral therapy (ART), people living with HIV (PLWH) suffer from a high burden of infectious and non-infectious pulmonary diseases, suggesting that their lung immunity is not fully restored. Cytotoxic CD8 T-cells are essential in controlling chronic viral infections. However, excessive CD8 T-cell activation during HIV infection can contribute to lung mucosal tissue damage. Furthermore, tobacco smoking is part of the lifestyle of many PLWH and smoking changes the lung environment, thus promoting pulmonary inflammation. Herein, we characterize the effects of HIV and smoking on cytotoxic tissue-resident memory (Trm) CD8 T-cell dynamics in the human lung.

Methods: Bronchoalveolar lavage (BAL) fluid and matched blood were obtained from asymptomatic ART-treated PLWH (median undetectable viral load: 8 years) smokers and non-smokers, and HIV-uninfected smokers and non-smokers (n=4-7/group). Lymphocytes were isolated and CD8 subsets were characterized by multiparameter flow cytometry. CpG methylation of the upstream PRF1 promoter was measured via bisulfite sequencing.

Results: Both smoking and HIV infection were independently associated with a significant increase in total CD8 T-cell frequencies in BAL. Their *PRF1* promoter region was largely demethylated compared to circulating naïve and memory CD8 T-cells. Within all study groups, CD69+ CD8 T-cell subsets were the most abundant, while the CD69- subsets were the least abundant. Furthermore, smoking, but not HIV status, was associated with a significant reduction of CD103-CD8 Trm subsets, as well as increased co-expression of CXCR6/CXCR3. Moreover, CD8 Trm from HIV+ versus HIV- study participants displayed higher levels of cytotoxic effector molecules granzymes A/B, with memory non-Trm cells also showing increased perforin expression.

Conclusions: Despite long-term ART, chronic pulmonary inflammation caused by HIV infection may dysregulate mucosal CD8 T-cell cytolytic functions. Smoking could promote increased CD8 T-cell migration via CXCR6-CXCL16 axis and their subsequent retention in the lung via CD103.

RÉSUMÉ

Contexte : Malgré le succès de la thérapie antirétrovirale (TARV), les personnes vivant avec le VIH (PVVIH) souffrent fréquemment de maladies pulmonaires infectieuses et non infectieuses, ce qui suggère que leur immunité pulmonaire n'est pas entièrement restaurée. Les lymphocytes T CD8 cytotoxiques sont essentiels dans le contrôle des infections virales chroniques. Cependant, une activation excessive des lymphocytes T CD8 au cours de l'infection par le VIH peut contribuer à endommager les tissus muqueux pulmonaires. De plus, le tabagisme est très fréquent au sein des PVVIH, chez qui il modifie l'environnement pulmonaire, favorisant ainsi l'inflammation pulmonaire. Ici, nous caractérisons les effets du VIH et du tabagisme sur la dynamique des lymphocytes T CD8 cytotoxiques mémoires, résidents dans le tissus pulmonaire humain (Trm).

Méthodes : Du liquide de lavage broncho-alvéolaire (LBA) et du sang ont été obtenus de PVVIH asymptomatiques sous TARV (durée médiane avec charge virale indétectable : 8 ans), fumeurs et non-fumeurs, et de fumeurs et non-fumeurs non infectés par le VIH (n = 4 à 7/groupe). Les lymphocytes ont été isolés et les sous-populations de CD8 ont été caractérisées par cytométrie en flux multiparamétriques. La méthylation CpG en amont du promoteur *PRF1* a été mesurée par séquençage au bisulfite.

Résultats : Le tabagisme et l'infection par le VIH étaient associés de manière indépendante à une augmentation significative des fréquences totales de lymphocytes T CD8 dans le LBA. Leur région promotrice PRF1 était largement déméthylée par rapport aux lymphocytes T CD8 circulants, naïfs et mémoires. Dans tous les groupes d'étude, les sous-populations de cellules T CD8 CD69+ étaient les plus abondantes, tandis que les sous-populations CD69- étaient les moins abondantes. De plus, le tabagisme, mais pas le statut VIH, était associé à une réduction significative des cellules CD8 Trm CD103-, ainsi qu'à une co-expression accrue de CXCR6/CXCR3. De plus, les CD8 Trm des participants à l'étude VIH + *versus* VIH présentaient des niveaux plus élevés de molécules effectrices cytotoxiques telles que les granzymes A et B, les cellules mémoire non Trm montrant également une expression accrue de la perforine.

Conclusions : Malgré un traitement antirétroviral à long terme, l'inflammation pulmonaire chronique causée par l'infection par le VIH peut dérégler les fonctions cytolytiques des lymphocytes T CD8 muqueux. Le tabagisme pourrait favoriser une migration accrue des lymphocytes T CD8 via l'axe CXCR6-CXCL16 et leur rétention ultérieure dans les poumons via le CD103.

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Lastly, I want to thank my mom for sacrificing almost everything she had when we moved to Canada and for enduring a very long and tough decade of immigrant life to give me a better future.

LIST OF ABBREVIATIONS

AIDS - Acquired Immune Deficiency Syndrome

AM - alveolar macrophage

ART - Antiretroviral therapy

BAL - bronchoalveolar lavage

CA - capsid

CMV - Cytomegalovirus

COPD - chronic obstructive pulmonary disease

CT scan - computed tomography scan

CTL - cytotoxic T-lymphocyte

CVIS - Chronic Viral Illness Service

DC - dendritic cell

FasL - Fas Ligand

HEV - high endothelial venules

HIV - Human Immunodeficiency Virus

HTLV - Human T-lymphotropic Virus

IFN - interferon

IL - interleukin

ILC - innate lymphoid cell

IN - integrase

KLRG1 - Killer cell lectin-like receptor subfamily G member 1

LTR - long terminal repeat

MA - matrix

MAIT - mucosal-associated invariant T-cell

MMP - matrix metalloproteinase

MSR - methylation-sensitive region

Mtb - Mycobacterium tuberculosis

MUHC - McGill University Health Centre

NC - nucleocapsid

NETs - neutrophil extracellular traps

NK T-cells - natural Killer T-cells

NLR - NOD-like receptor

Non-Trm - non-tissue resident memory T-cells

NS - non smoker

PBMC - peripheral blood mononuclear cells

PLWH -People living with HIV

PNAd - Peripheral node addressin

PrEP - pre-exposure prophylaxis

ROS - reactive oxygen species

S1P - spingosine-1-phosphate

S1PR1 - spingosine-1-phosphate receptor 1

SARS-CoV-2 - severe acute respiratory syndrome coronavirus 2

SIV - Simian Immunodeficiency Virus

SM - smoker

Tfh - follicular helper T-cell

TGF-β - transforming growth factor β

TLR - Toll-like receptor

TNFR1 - tumor-necrosis factor-receptor 1

TNF-α - tumor-necrosis factor-α

Tregs - regulatory T-cell

Trm - Tissue-resident T-cells

UNAIDS - Joint United Nations Programme on HIV/AIDS

UQAM - Université du Québec à Montréa

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PREFACE

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

Author contribution for Chapters 1-7 of Literature Review:

These chapters were written by Yulia Alexandrov, with suggestions from Dr. Cecilia T. Costiniuk and Dr. Mohammad-Ali Jenabian.

Author contribution for Chapter 8 Methods:

The methodologies described were designed by Yulia Alexandrova, Dr. Cecilia Costiniuk, Dr. Mohammad-Ali Jenabian and Tao Shi.

Author contribution for Chapter 9 Results:

The experiments were conducted by Yulia Alexandrova and Tao Shi.

Analyses and interpretation of the data were performed by Yulia Alexandrova, Dr. Cecilia Costiniuk, and Dr. Mohammad-Ali Jenabian.

Author contribution for Chapters 10 Discussion and Chapter 11 Future Directions:

The chapter was written by Yulia Alexandrova, with suggestions from Dr. Cecilia T. Costiniuk and Dr. Mohammad-Ali Jenabian.

CHAPTER 1: HIV & AIDS

1.1.1 Discovery of HIV

In July of 1981, an article in Morbidity and Mortality Weekly Report (MMWR) came out titled "Kaposi's Sarcoma and *Pneumocystis* Pneumonia in Homosexual Men" describing a rising number of cases of previously uncommon malignancies suddenly appearing in young gay men in New York and California[1]. Patients presented with skin and oral mucosal lesions, pneumonia, weight loss, fever, and swollen lymph nodes. Some even experienced severe recurrent herpes simplex infection, extensive candidiasis, and cryptococcal meningitis. Because the disease was most common in gay men and injection drug users, it became stigmatized and unfortunately the fear of "*gay plague*" spread across the United States[2].

The term AIDS (Acquired Immune Disease Syndrome) was used for the first time in September of 1982 by Center of Disease Control[2]. Careful immunological analysis provided researchers with clues about the true cause of the disease. A key observation made during disease progression was a large drop in circulating CD4 T-cells. If a patient's CD4 T-cell count fell below 200 cells/mm^3, he or she became susceptible to a myriad of opportunistic infections and previously rare malignancies. The same T-cell subset was previously known to be targeted by Human T-lymphotropic virus 1 (HTLV-I), the first human retrovirus to be described in 1980 by Dr Robert Gallo and his colleagues[3]. However, instead of decreasing CD4 T-cell numbers, HTLV-I infection led to T-cell leukemia[4,5]. This led scientists to suspect that the disease was caused by a T-cell tropic retrovirus. Heterosexual transmission of the disease was first documented in January 1983 MMWR issue[6]. It became clear that this disease has already spread like wildfire in countries other than the US, including Europe and Sub-Saharan Africa, the latter bearing the higher burdens of infections rates, and soon engulfing the whole world (Figure 1). Moreover, the image of the "*gay plague*" began to crumble, since the worldwide transmission pattern was heavily leaning towards the spread among heterosexual partners[2]. In 1983, Dr Barre-Sinoussi and colleagues were first to isolate the novel retrovirus from a lymph node of a pre-AIDS patient. The isolate proved to be rather different from HTLV-I[7]. In 1986, the International

Committee of the Taxonomy of Viruses finally gave it its own proper name– Human Immunodeficiency Virus (HIV)[8].

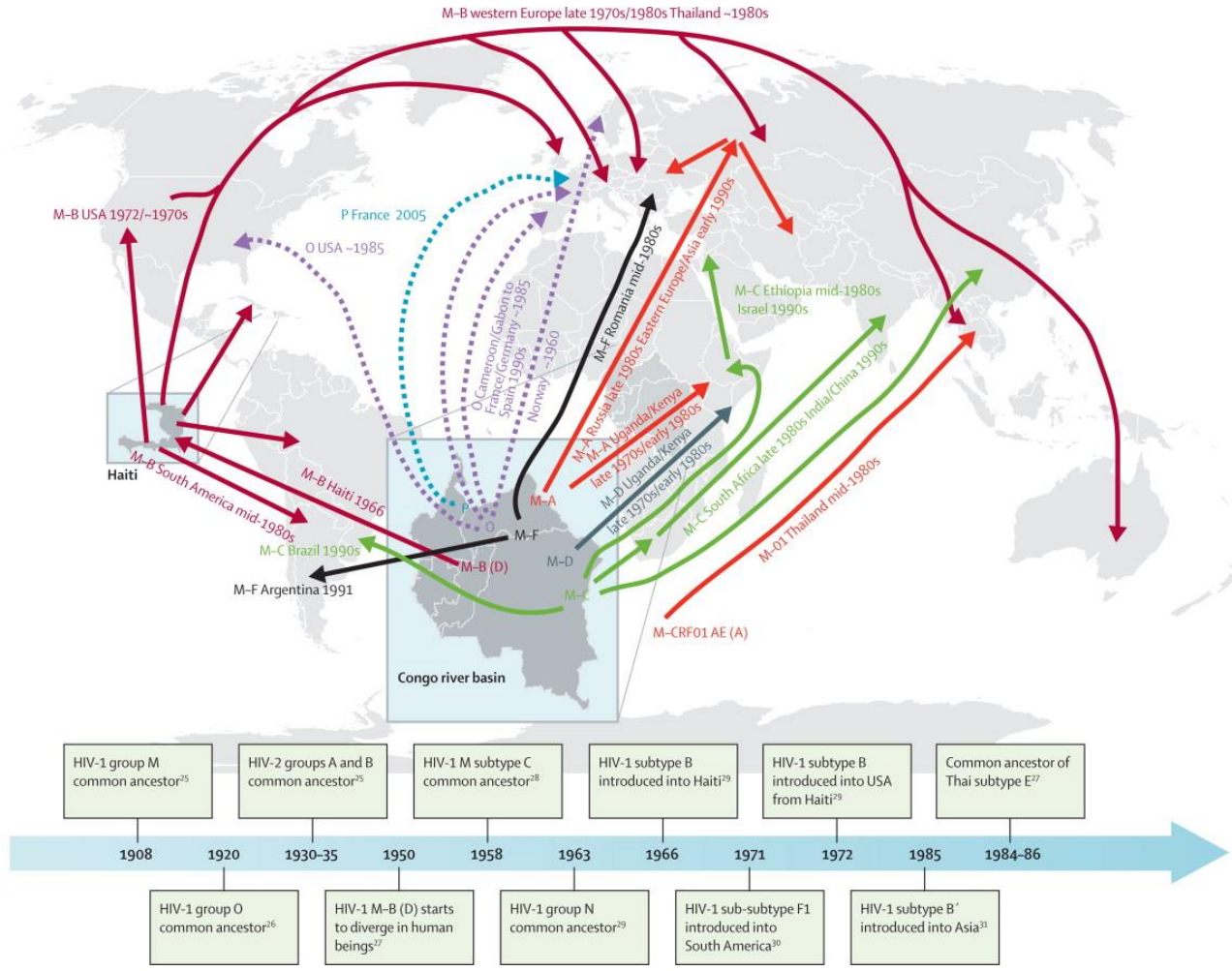

Figure 1: Evolution and spread of HIV groups and subtypes. Disease epicenters are enlarged. Key transmission events of HIV-1 groups M, N, and O and of HIV-2 are indicated on the timeline[9].

Phylogenetic studies suggest that HIV originated in the Democratic Republic of Congo during the 1920s, when the Simian Immunodeficiency Virus evolved and crossed species from chimpanzees to humans[10,11]. Notably, several different HIV subtypes arising from multiple cross-species events have been previously documented. However, their spread was quite limited[12]. HIV-1 group M, the principal cause of the AIDS pandemic, was an exception. Studies suggest that its common ancestor appeared during a Simian Immunodeficiency Virus (SIV)

transmission event from chimpanzees in southeastern Cameroon, spreading to Leopoldville in Congo – the largest city in the region[10,11]. It then disseminated across the world throughout the 20th century[12,13]. Some researchers propose that HIV-1 group M's common ancestor dates all the way back to the 1920s, meaning that HIV was spreading for at least 50 years before it was discovered[13]. The true evolution and spread of HIV, however, remain a mystery since there is no historical record dating back to its time of origin. Thus, these proposed evolutionary timelines should only be considered as a speculation.

1.1.2 Epidemiology of HIV

HIV remains to be one of the major global health issues up to this day. According to Joint United Nations Programme on HIV/AIDS (UNAIDS) report published in 2021, there were 37.7 million people living with HIV (PLWH). Eastern and Southern African regions continue to bear the world's highest HIV infection rates, with 20.6 million PLWH, which accounts for two thirds of all HIV infections globally[14]. In Western and Central Africa the estimated number of PLWH is 4.7 million. Africa is followed by Asia and the Pacific in HIV infection burden (5.8 million PLWH), Western and Central Europe with North America (2.2 million PLWH), Latin America (2.1 million PLWH), and Eastern Europe with Central Asia (1.6 million PLWH). Common routes of transmission vary among these regions. The virus is primarily transmitted through heterosexual contact in Sub-Saharan Africa, injection drug use in Eastern Europe, Central Asia, North Africa, and the Middle East, and men having sex with men in North America and Western Europe[15].

HIV is especially prevalent among vulnerable populations all over the world, especially in countries where these populations are marginalized. Gay men and other men having sex with men are 25 times more likely to be infected than heterosexual men. Female sex workers are at 26 times greater risk of getting HIV than women in the general population. Transgender women are at 34 times greater risk than other adults. Injection drug users are 35 times more likely to be infected than non-users[14]. In 2019, 65% new adult HIV infections globally were among these at-risk populations, accounting for 93% of new HIV infections outside of sub-Saharan Africa and 39% of new HIV infections in sub-Saharan Africa[16]. Moreover, criminalization of these populations is correlated with fewer PLWH being aware of their status and fewer people having

suppressed viral loads, slowing down the global HIV response and leading to poorer HIV outcomes[17].

Multiple campaigns have been organized during the past decade to combat the HIV crisis. The one launched by UNAIDS Programme Coordinating Board is the most ambitious than any of its predecessors: the "90-90-90 Treatment Target" and eradication of AIDS by 2030[18]. The campaign's objective is to ensure that 90% of infected individuals get tested and become aware of their status, 90% of those receive anti-retroviral treatment (ART), and that 90% of the treated patients become virally suppressed. When complete viral suppression is achieved the infected individual is no longer able to transmit the virus. UNAIDS' tremendous effort has inarguably helped curb global HIV infection rates, which have dropped by 31% since 2010 (Fig.2). In 2020, their 90–90–90 targets were not too far from their goal: 84% of PLWH knew their status, 87% of those had access to ART, and 90% of PLWH on ART were virally suppressed[14]. However, these are only gross estimates, since the true denominator for these measurements is unknown.

While HIV infection rates in most countries continue to drop, that is not the case for Eastern Europe and Central Asia, where infection rates are still rising. This is partially due to the afore mentioned punitive government measures that target these vulnerable populations, especially men who have sex with men and injection drug users, leading to reduced prevention and treatment access for these individuals[19].

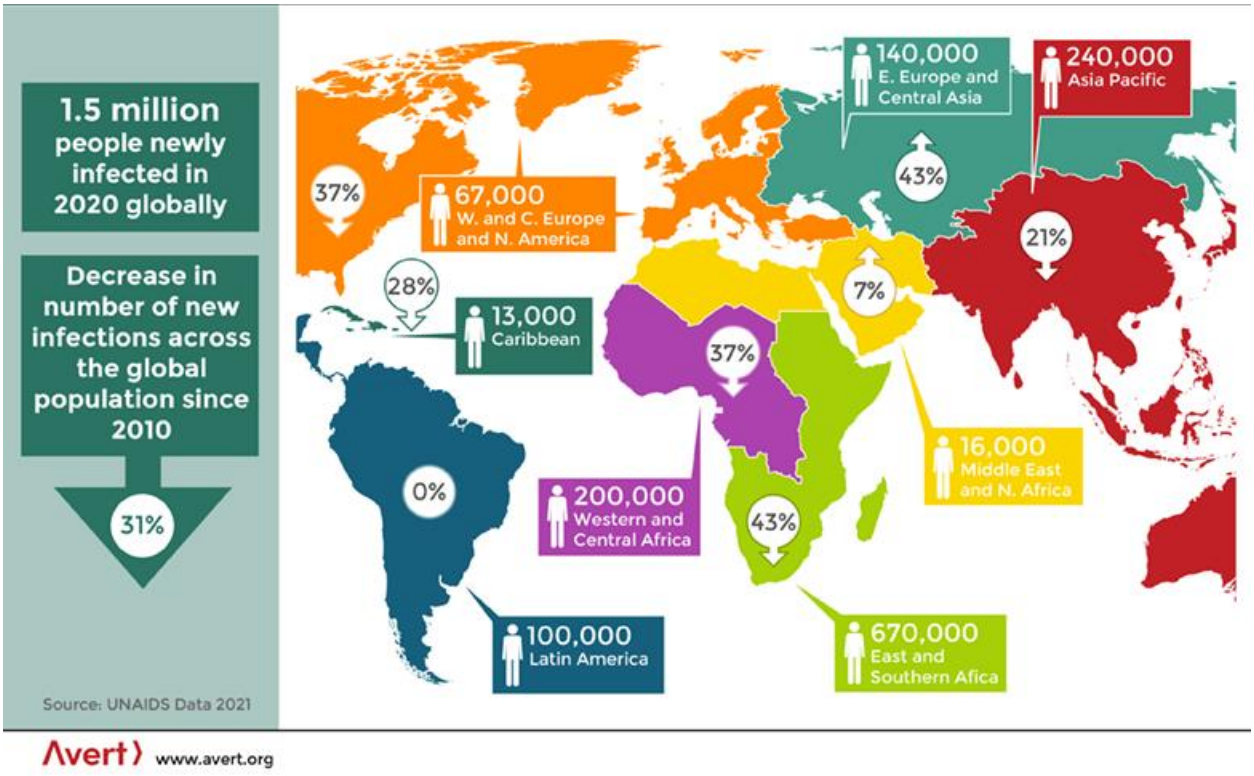

Figure 2: New HIV infections in 2020 and change since 2010. HIV infection rates in most countries have dropped considerably. However, that is not the case for Eastern Europe and Central Asia, where infection rates are still on the rise[14,19].

1.1.3 HIV Life Cycle

Viral structure and genome:

HIV-1 and HIV-2 are both lentiviruses from the viral family of *Retroviridae*. As retroviruses, they possess the ability to convert their RNA genome into DNA, with the help of reverse transcriptase (RT), and insert in into the genome of the host cell[20]. HIV virions contain two copies of their genome as linear single stranded positive sense RNA. These encode four Gag proteins (MA (matrix), CA (capsid), NC (nucleocapsid), and p6), and two Env proteins (gp120 gp41), which make up the core and the envelope of the viral particle. In addition to these structural components, HIV genome encodes six accessory proteins (*vif, vpr, vpu/vpx, tat, rev, nef*)[20] (Fig.3). Lastly, each virion also contain three Pol proteins (PR (protease), RT, and IN (integrase)). HIV is a highly complex retrovirus compared to some of its simpler relatives in

Retroviridae. While simple retroviruses make just two mRNAs (unspliced and singly spliced), HIV full-length primary transcript can generate more than 20 different mRNAs that fall into three different classes: unspliced (9kb), singly spliced (4kb), and doubly spliced (2kb)[21].

HIV entry:

HIV attacks key effector cells of the human immune system: CD4 T-cells and macrophages. HIV virions use their Env protein, made of gp120 and gp41 subunits, to bind to the CD4 viral receptor expressed on these target cells. gp120 then undergoes a conformational change, giving it the ability to bind to a co-receptor, either CCR5 or CXCR4. Viruses that use CCR5 are termed R5-tropic, whereas those using CXCR4 are called X4-tropic[22]. Only the R5 strain can be sexually transmitted[23]. This binding leads to viral entry into the host cell via fusion of the viral envelope with the cell's plasma membrane, releasing the nucleocapsid into the cytoplasm.

HIV DNA integration:

Once the nucleocapsid has been released into the cytoplasm, it partially breaks down to access the cell's nucleotide pool and attaches itself to cellular microfilaments[24-26]. The viral genome is then converted into DNA through reverse transcription using cellular transfer RNA as primers at the 5' end of the viral genome[27]. Importantly, reverse transcription is a highly erroneous process due to RT's inefficient proofreading mechanisms, giving the virus a significant advantage when adapting to evolutionary pressures exerted by the hostile immune environment[27,28]. Once reverse transcription is complete, the DNA-protein complex becomes integration competent and is termed the preintegration complex.

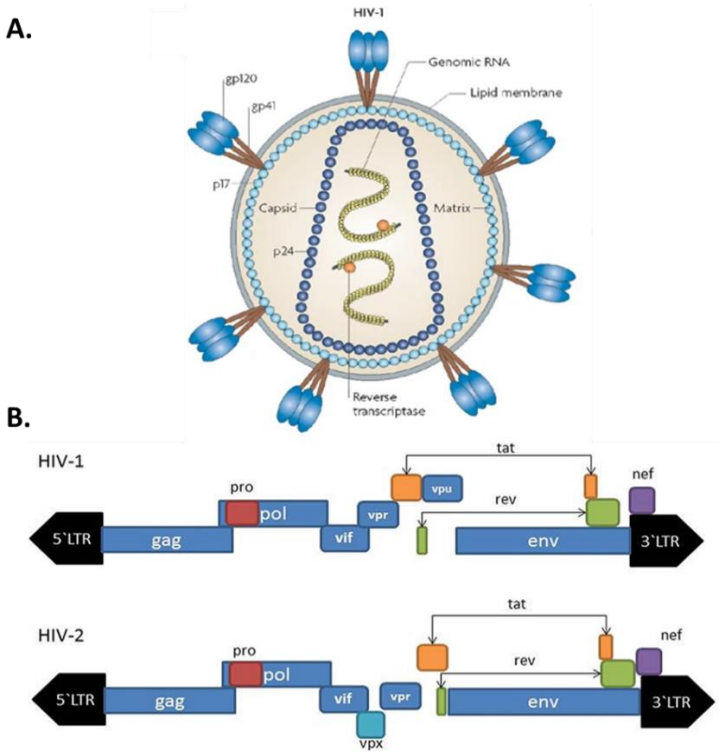

Figure 3: (A) Overview of HIV-1 structural components[29]. (B) Comparison of HIV-1 and HIV-2 genomes. Note that HIV-1 contains the accessory protein gene *vpu*, while HIV-2 contains *vpx* instead[30].

Three viral proteins facilitate preintegration complex transport: MA, Vpr, and IN. While the MA encodes the nuclear import signal, Vpr and IN interact with the nuclear pore for translocation and integration of the viral genome[31]. However, this occurs only upon T cell activation, whereas in resting T cells the virus fails to complete reverse transcription and is thus unable to integrate into the host genome[32]. Upon HIV integration, the infection will either become latent, where viral transcription is silenced, or active, where the infected cell actively expresses viral RNA and proteins. Notably, around 95% of all integrated proviruses are defective and only a small fraction remain replication competent[33,34].

Latent HIV infection

Latently infected cells pose by far the biggest hurdle on our way to an HIV cure. Although these cells do not directly contribute to AIDS progression, they continue to serve as viral reservoirs and cannot be eliminated by the immune system due to lack of viral antigens for it to recognize. This is also the primary reason why ART cannot cure HIV, which is discussed in more details in

later sections of this chapter. HIV latency is controlled by transcriptional elements present in HIV long terminal repeats (LTRs). U3 region of the HIV-1 LTR contains numerous binding sites for transcription factors, such as NFkB and NFAT, which have been described as positive regulators of HIV transcription initiation[35-37]. NFkB and NFAT are also highly sensitive to external stimuli, such as T-cell receptor activation or cytokine signaling. HIV core promoter contains a TATA box and an initiator element at the transcription start site, and the TAR sequence encoding an RNA stem-loop structure directly bound by Tat. The key function of this structure is recruitment of a cellular elongation factor complex that stimulates efficient transcription elongation[35,38]. Notably, HIV genome does not encode any transcriptional repressor proteins. Instead, HIV latency is controlled via restriction of Tat expression, thus hindering viral transcription and elongation[35] (Fig.4).

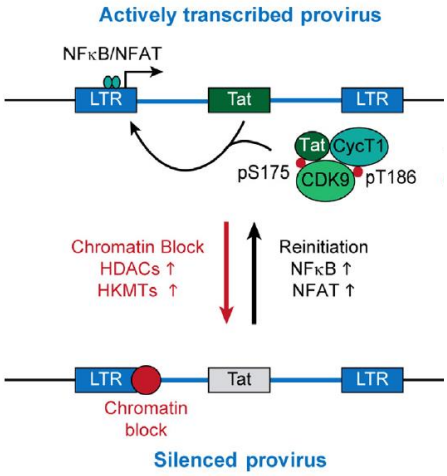

Figure 4: HIV promoter inactivation is largely caused by epigenetic silencing via histone deacetylases (HDACs) and histone lysine methyltransferase (HKMTs), which drives down Tat expression leading to HIV latency. In contrast, stimulation of transcription initiation induces Tat production, which further stimulates efficient viral transcription elongation[35].

HIV replication and packaging:

Viral proteins Tat and Rev are essential for viral replication. Expression of Tat dramatically increases the amount of RNA produced by infected cells. It does so by binding the TAR sequence of the nascent HIV RNA transcript and stimulating elongation through increased phosphorylation levels of the carboxy terminal domain of RNA polymerase II via cyclin-

dependent kinase 9/cyclin T recruitment[35]. Viral unspliced and singly spliced mRNAs, encoding Gag, Gag/pol, Env, Vif, Vpr, and Vpu, are in turn transported from the nucleus into the cytoplasm by Rev for translation[39]. Notably, doubly spliced HIV mRNA translation, which encode Tat, Rev, and Nef, is not dependent on Rev-mediated transport[40].

While HIV Env glycoproteins are synthesized in the rough endoplasmic reticulum, trafficking to the Golgi and then to the plasma membrane, both Gag and Gag/pol polyprotein precursors are synthesized in the cytosol. While the Gag polyprotein comprises of the matrix (MA), capsid (CA), nucleocapsid (NC) and p6 domains, the GagPol polyprotein contains MA, CA, NC, protease, reverse transcriptase and the integrase. As Gag begins to form multimers, it also recruits viral RNA to the plasma membrane and anchors itself in lipid raft microdomains. The assembling viral particle then incorporates Env and recruits the Endosomal Sorting Complex Required for Transport (ESCRT), which is needed for viral budding and membrane scission. Maturation of the viral particle is mediated by the viral protease, which cleaves Gag and GagPol polyprotein precursors into individual protein subunits[41] (Fig.5).

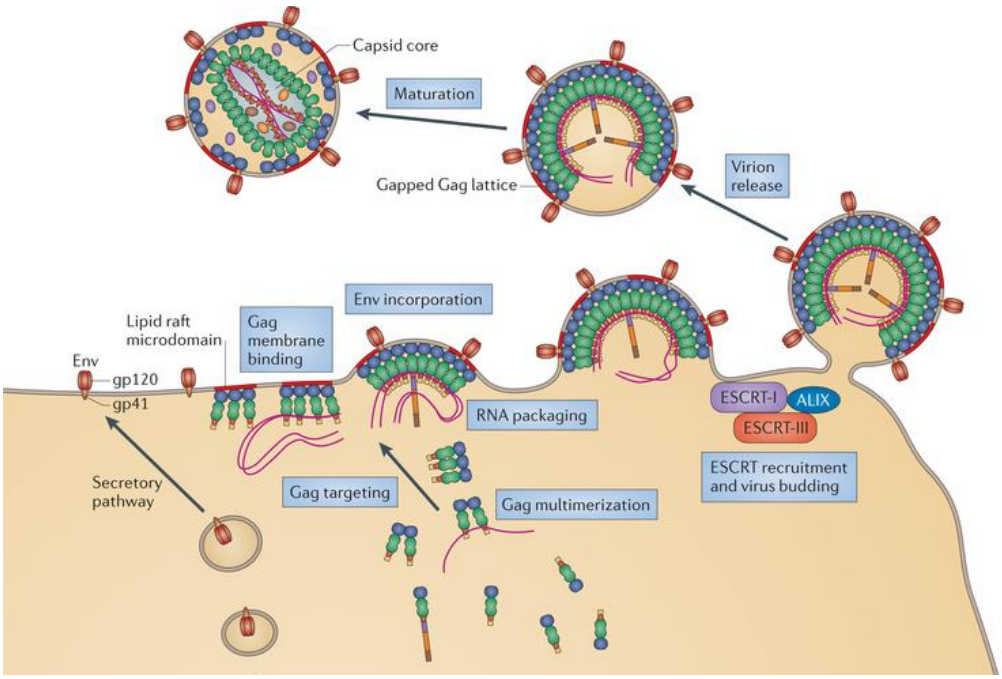

Figure 5: Overview of HIV-1 viral particle assembly[41].

1.1.4 HIV Disease Stages

HIV is not an airborne pathogen and can be transmitted only through certain bodily fluids. These include blood, semen, rectal and vaginal fluids, and breast milk, which are exchanged during very specific activities, such as sexual behaviours, syringe use, and blood transfusions[42]. HIV's main target cells, most permissive to infection, are CD4 T-cells. In case of sexual contact, HIV moves across the epithelial barrier, coming into contact with mucosal CD4 T-cells. These infected cells begin to produce and disseminate virus across the lymphoid system and eventually the blood, spreading it further to other tissues[43,44]. To enter the cell, the HIV viral particle binds to CD4 molecule on the cell surface - its main receptor. It also must bind to either CCR5 or CXCR4, which act as co-receptors depending on the HIV genetic variant. CCR5-tropic HIV can infect central memory CD4 T-cells and macrophages, while CXCR4 tropic virus can infect both central memory and naïve CD4 T-cells, although the naive subset is much less susceptible to infection due to lower viral fusion efficiency compared to central memory cells[45-47].

Primary infection is followed by Fiebig stages I-V, which are based on a series of HIV-1 clinical diagnostic assays that test positive in a stepwise manner (Fig.6)[48]. Primary infection is followed by an eclipse period, where none of the viral markers are detectable. The eclipse period can last up to 11 days, and is followed by stage I: HIV RNA viremia, where only the RNA assay tests positive. Stage II is p24 seroconversion: levels of p24 cross the minimal threshold of detection during the 13 days after viral RNA tests positive. On average, 5 days after p24 seroconversion the infection reaches stage III, where HIV IgM response can be detected by an enzyme immunoassay. During stages IV and V, other viral antigens, such as p17, gp41, gp120, and gp160, show up on the Western Blot. Stage IV manifests in an indeterminate Western Blot result, where the number of viral protein bands are insufficient to meet the interpretative criteria defined by the USA Food and Drug Administration. In contrast, stage V meets all of the requirements for a positive HIV Western Blot test, around the same time as blood viremia reaches its peak. According to the study carried out by Kong *et al.*, the mean time for a complete Western Blot seroconversion, is ~44 days[48,49].

Symptom onset begins during the acute phase ~2 weeks after primary infection, which coincides with peak viremia[50,51]. At this point, viral reservoirs are established within long lived

cells, other than CD4 T-cells[52-55]. Around 100 days following HIV exposure, the infection approaches the end of stage V and progresses from acute to an early chronic phase.

During the chronic stage, the viral load remains relatively low and stable for many years thereafter. Without treatment, the virus will slowly deplete the body's CD4 T-cell counts, followed by an increase in overall memory T-cell turnover, thymus and lymphoid tissue damage, and physiological restrictions in renewal of peripheral CD4 T-cells, thus compromising the patient's immune system in face of secondary infections and cancers. This condition is widely known as Acquired Immunodeficiency Syndrome (AIDS), a disease that is characterized by a drop in CD4 T-cell count to <200 cells/µl, which can take up to 10 years to develop[56,57].

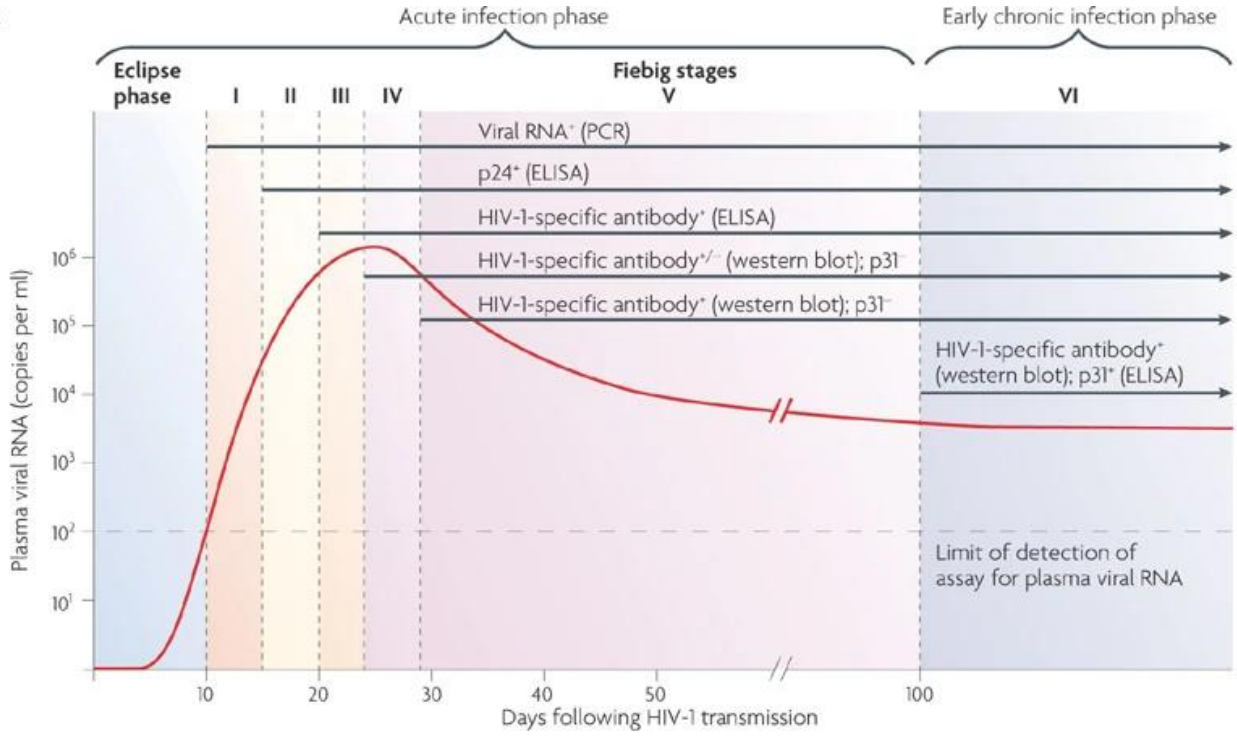

Figure 6: Overview of Fiebig stages I–VI. HIV infection stage can be determined based on a positive HIV-1 clinical diagnostic assays: viral RNA measured by PCR, viral antigens measured by enzyme-linked immunosorbent assay (ELISA), or HIV-1-specific antibodies detected either by ELISA or western blot)[43].

1.1.5 HIV Viral Reservoirs

Despite the success of ART at inhibiting HIV replication, discussed in a later section, the major challenge for a functional HIV cure is the viral persistence in cellular and tissue reservoirs. An HIV reservoir is an anatomical site harboring cells where a replication-competent proviral DNA with stable kinetic properties persists in its integrated form[57]. After acute infection, the provirus enters a state of post-integration latency whereby viral transcription is reversibly silenced. The lack of viral protein expression on the cell surface protects the cell from detection and elimination by the immune system[58]. Cellular HIV reservoirs are established during the early days of infection, which include CD4 T-cells and macrophages, whereby HIV seeds different anatomical sites such as lymph nodes, gut-associated lymphoid tissue, the central nervous system, the genitourinary tract, and the lung (Fig.7) [59-61].

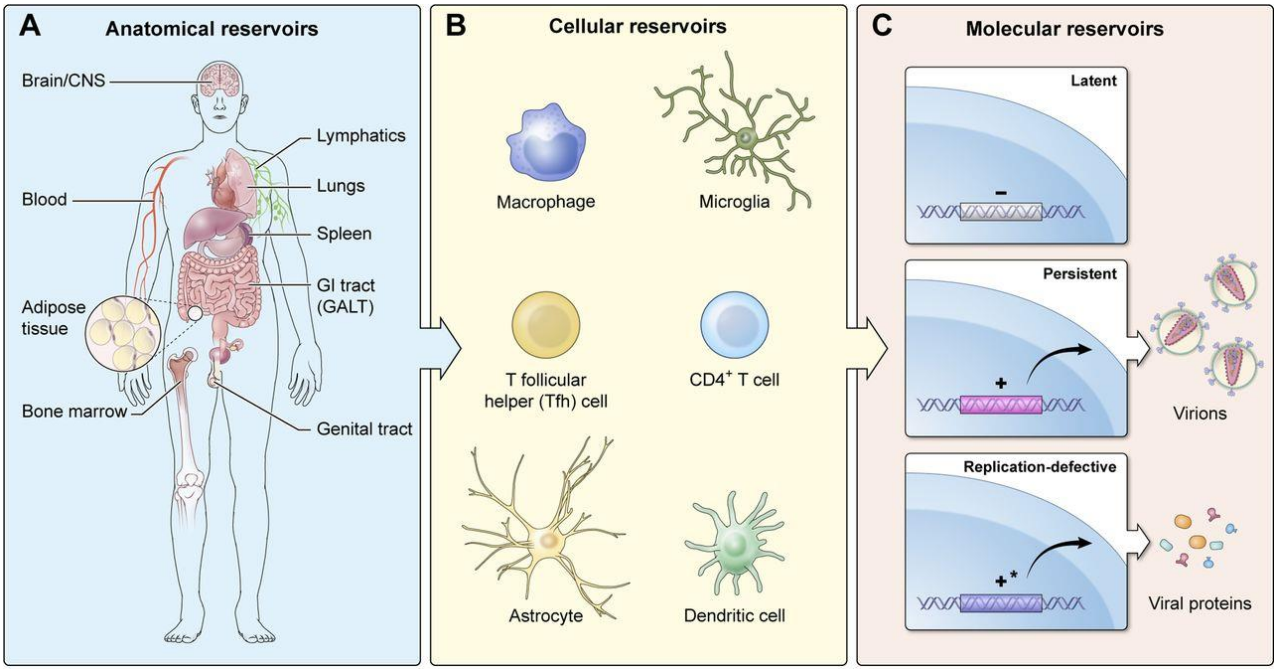

Figure 7: Overview of HIV-1 anatomical, cellular, and molecular reservoirs. Note that because the immune system spans the entire human body, so does the virus upon infection[59,62].

Several suggested mechanisms have been put forth to explain the manner in which HIV reservoir persists inside the body even after many years of ART treatment, some of which have been mentioned earlier. Such mechanisms include poor ART penetration into deep tissues, residual viral replication, persistent cell stimulation due to residual antigen load, and CD8 T-cell exhaustion[63,64]. All these mechanisms are likely closely intertwined. If deep tissue reservoirs

are not fully inhibited by ART compounds, some of the virus will continue replicating, increasing viral antigen load locally[65,66]. In turn, this antigen load will stimulate nearby immune cells, activating their transcriptional machinery, which will produce more virus if that cell harbors intact and inducible viral DNA. Several studies have shown a correlation between levels of immune activation and HIV reservoir size, further confirming their interdependence[67,68].

1.1.6 Antiretroviral Therapy

ART has been very successful in transforming HIV from a death sentence into a manageable chronic condition and at limiting its sexual transmission[69]. The pharmaceutical compounds used in combinations in ART regimens act by inhibiting different stages of the HIV life cycle. These include nucleoside and non-nucleoside reverse transcriptase inhibitors, protease inhibitors, and integrase inhibitors[70] (Fig.8). Although ART leads to partial restoration of CD4 T-cell counts[71], its major pitfall is that it only inhibits viral replication but does not eliminate the infection[72,73]. Firstly, ART has variable penetration into different tissues, and secondly, long-lived HIV latently infected cells are inherently resistant to elimination by our immune system[74,75]. It is important to keep in mind that various ART regiments either prevent viral exit from already infected cells or prevent further cell to cell spread by blocking viral entry. Once the virus has integrated its genome and entered the latent phase, all ART can do is prevent active viral amplification and spread. ART interruption almost inevitably leads to viral rebound, even after patients receive stem-cell transplants and early treatment. The Mississippi infant and two HIV-infected patients in Boston, USA who received cell transplants for lymphoma all had undetectable viral load prior to ART interruption, and yet only transient remission was achieved[76].

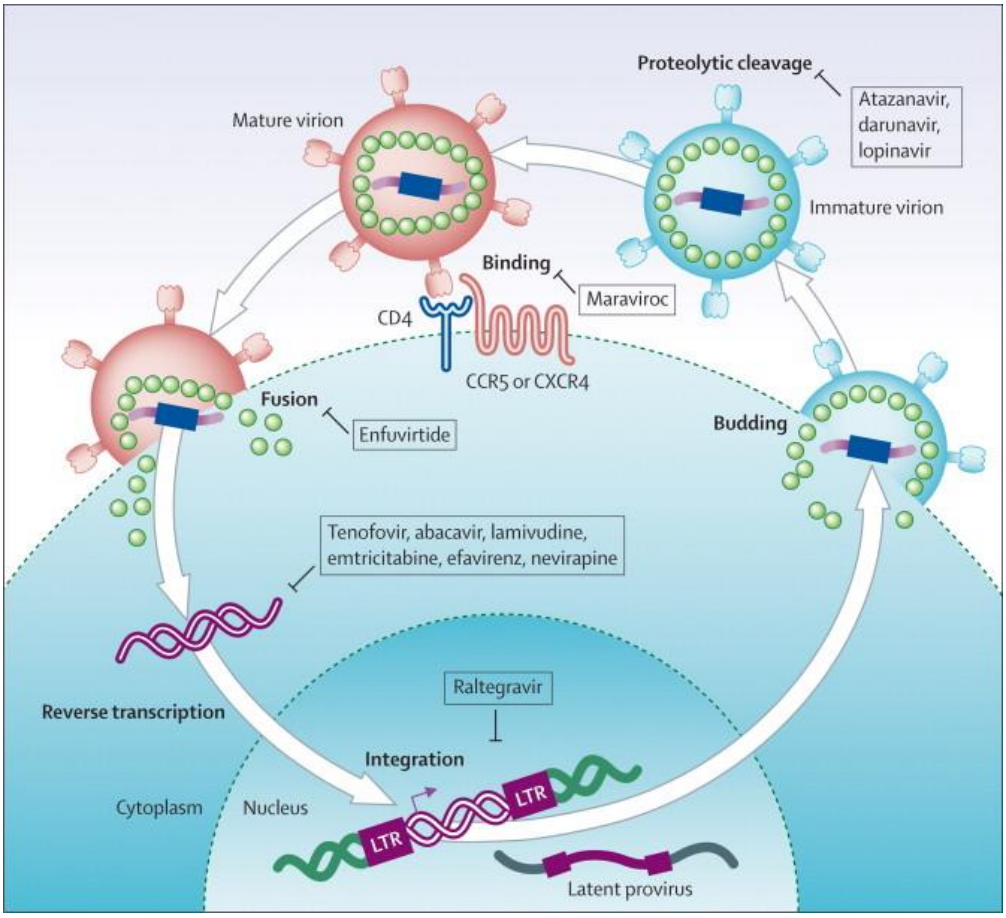

Figure 8: Examples of drugs targeting different stages of HIV-1 replication cycle[77].

HIV-1 infection is characterized by chronic immune activation and inflammation, which are predictors of disease progression[43,78]. In fact, HIV-positive status has been linked to higher prevalence of age-associated noncommunicable comorbidities, such as cardiovascular and renal disease, where inflammation is believed to be one of contributing factors[79]. Furthermore, chronic inflammatory states persist in treated PLWH despite virologic suppression with ART, which has been linked to a higher non-AIDS related morbidity and mortality rate [80,81]. The prevalence of infectious and non-infectious comorbidities is also pertinent regarding the lung. Even on ART, PLWH are 25 times more likely to suffer from pneumonia and are at a higher risk of developing chronic obstructive pulmonary disease or lung cancer than healthy individuals[82-86]. Rates of tuberculosis infection and influenza-associated mortality is also higher in PLWH than in general population[87,88]. More details on persistent lung immune dysfunction in PLWH on ART are further covered in a later section.

CHAPTER 2: Lung Immunity

Just like the skin and the gastrointestinal tract, the lungs are an interface between the inner body and the outside world. Apart from ensuring adequate gas exchange, the lung must prevent harmful effects of noxious compounds, microbes, and debris on the body. The lungs are designed to facilitate optimal oxygen exchange between the environment and red blood cells. Thus, not only are they highly vascularized, but they also house around 300 million alveoli, covering a surface area of approximately 500 m^2 – roughly the size of a tennis court[89,90]. This entire area must be kept clean of unwanted airborne particles and is patrolled continuously for foreign invaders – a task executed by the innate and adaptive arms of the immune system.

2.1 Physical and Biochemical Barriers

The human respiratory tract can be divided into the upper and lower parts. The upper portion is composed of the nasal cavity, pharynx, and larynx, while the lower portion includes the trachea and the lung itself, which houses part of the conducting airways (bronchi, bronchioles) that lead into the respiratory zone (the alveoli) (Fig.9)[91]. The lumen of the airways is lined with epithelium that is made up of ciliated, non-ciliated, and secretory cells[91]. These are sealed shut with tight junctions, creating a physical barrier, whose permeability is controlled by transmembrane proteins called claudins[92]. This barrier is further reinforced by a layer of airway surface fluid made of mucins - large heavily glycosylated proteins secreted by goblet cells that form a gel-like physical barrier that protects the underlying cells from physical and chemical stressors. Moreover, this barrier acts as an antimicrobial wall, trapping any microbes with which it comes into contact[93]. In addition to mucins, airway surface fluid contains a large number of antimicrobial proteins and peptides, such as defensins and lysozymes, which collectively exhibit broad-spectrum antimicrobial activity[94]. Airway mucous, along with the trapped microbes and debris within it, is pushed out continuously from the lower lung into the trachea and esophagus by the mucociliary clearance system where ciliated cells move the surface fluid via the motive force of their cilia. As we progress from the terminal bronchus into the alveolar sacs, the

cellular and airway surface fluid makeup changes. Here, in the alveolar sacs, the epithelial barrier becomes

extremely thin and its surface mucus becomes replaced by surfactant, a detergent-like substance that prevents alveolar collapse when we breathe[95]. Since gas exchange in this zone is vital, structural damage, air flow obstruction, or uncontrolled inflammation within this tissue can have life-threatening consequences[96].

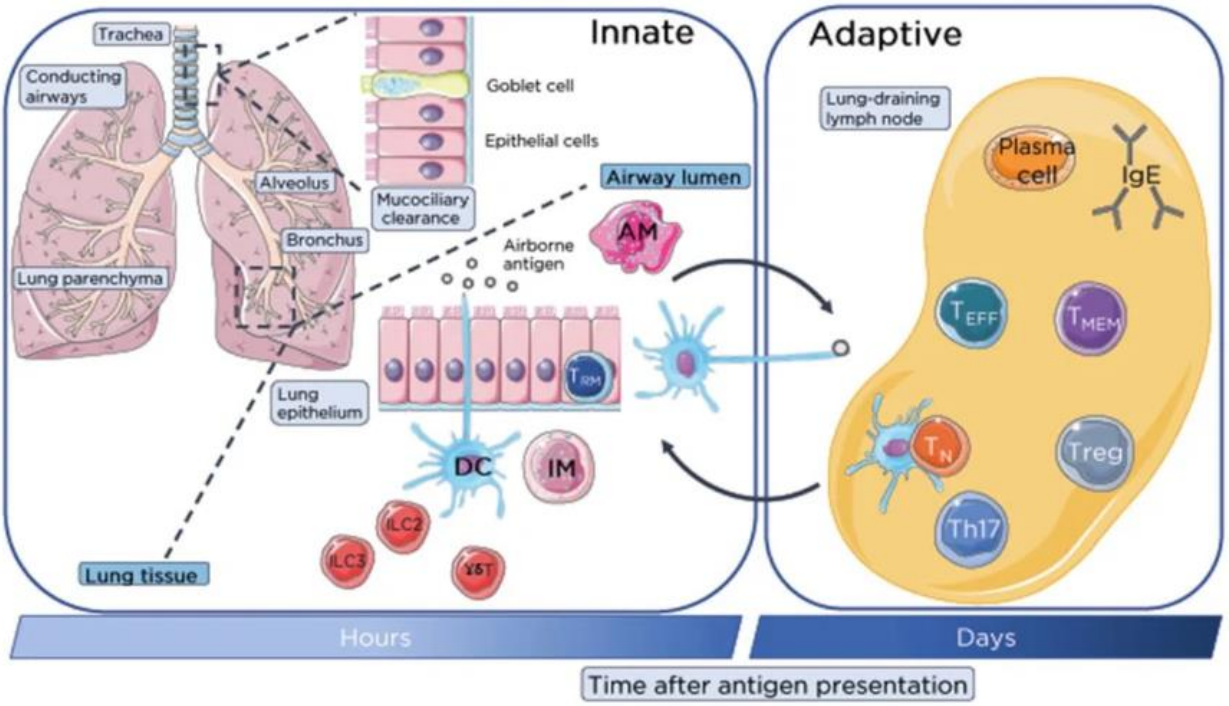

Figure 9: Summary of the lung immune cell network. Lung tissue-resident cells and the mucociliary clearance help maintain immune homeostasis. Alveolar macrophages (AM) clear foreign antigens and debris and receive multiple inhibitory signals from pulmonary epithelium that prevent their unnecessary activation. Other immune cells, like innate lymphoid cells (ILCs) and tissue-resident memory T-cells (Trm) provide a rapid first-line of defence to inhaled antigenic particulates. Microbes that cannot be cleared by these cells are sampled by dendritic cells (DCs), which then migrate to the lung-draining lymph nodes looking for a matching T-cell. Activated antigen-specific T cells undergo proliferation and migrate to the site of infection[97].

2.2 Sensor Cells of the Innate Immune System

The next tier of the lung's defense system are sensor cells, which include the aforementioned epithelial cells along with alveolar macrophages (AMs), dendritic cells (DCs), and mast cells (Fig.10)[95]. In bronchoalveolar lavage (BAL) fluid, AMs make up the majority of this cell pool (~85%)[98]. Unlike most immune cells, AMs are largely derived from unique tissue-resident cell subset that originates from a distinct hematopoietic cell lineage during embryonic development[99,100]. When quiescent, the primary role of AMs is to clear the lung of allergen particles, dead cells, and other debris to maintain tissue homeostasis. They also receive multiple inhibitory signals, such as CD200, transforming growth factor-β (TGF-β) and interleukin-10 (IL-10), from pulmonary epithelium that prevent their unnecessary activation[101]. Notably, healthy lung microbiota further supports these anti-inflammatory homeostatic functions[102]. However, during an active infection, these processes are interrupted. Once a pathogen is encountered and recognized by one of the aforementioned sensor cells, pro-inflammatory cytokines are released, which immediately initiate an innate immune response. The type of immune response initiated depends on the nature of the pathogen and the type of cytokines it triggers. For instance, in the case of a viral infection, epithelial cells, AMs and DCs begin producing Type I and Type III interferons (IFNs), to limit pathogen spread and induce an antiviral state of the cells in the vicinity[103].

2.3 Engaging the Adaptive Immune System

If the aforementioned mechanisms fail to clear the pathogen, tissue-resident lymphoid cells are recruited (Fig.10). These include innate lymphoid cells, natural killer cells, Natural Killer (NK) T-cells, conventional CD8 and CD4 T-cells, as well as mucosal-associated invariant T-cells (MAIT) and γδ T-cells. Collectively, these cells further enhance direct killing of the pathogen or infected cells and recruit more effector cells from the circulation, such as neutrophils and monocytes, to facilitate infection clearance in its early stages[95]. If the pathogen is still not cleared, additional forces of the adaptive immune system are called to action. DCs are the key mediators of this process. Residing under the epithelial layer within the pulmonary interstitium, these cells can extend their dendrites across the epithelial layer to sample antigen from the lumen. Under

inflammatory conditions, DCs become activated and begin to transport the microbial antigen to the draining lymph nodes and nearby mucosal associated lymphoid tissues, where they activate naïve and central memory T-cells whose T-cell receptor matches the MHC-peptide complex on DC's surface[104-106]. The efficiency of DCs to activate these cells depends on both the nature of infection and the type of T-cell it encounters. For example, during respiratory influenza virus infection, CD103+ migratory DCs are the most potent activators of naïve virus-specific CD8 T-cells[107,108].

CD8+ cytotoxic T-cells and CD4+ Th1 cells fight intracellular microbes by killing infected cells, releasing pro-inflammatory and anti-viral cytokines (IFN-γ), and recruiting phagocytes to the infection site[95,104,109]. CD8+ Tc2 cells and CD4+ Th2 cells fight extracellular parasites via granulocyte recruitment, mast cell activation, stimulation of mucus production by goblet cells, and promotion of B-cell class-switching to IgE[95,109]. CD4+ Th17 and CD8+ Tc17 cells are devoted to battling against extracellular bacteria and fungi. These cells amplify neutrophil recruitment, stimulate antimicrobial peptide production by the pulmonary epithelium, and promote B-cell class-switching to opsonizing antibody production[109,110]. B-cells and follicular helper T-cells (Tfh) are crucial against all classes of pathogen. With the aid of Tfh cells, B-cells expand and differentiate. Depending on the activation site and signals they receive from Tfh cells, some will traffic back to the airways and become local IgA producing plasma cells, while others will switch to IgG and home to the bone marrow to provide systemic protection[111]. Notably, in a healthy respiratory tract, IgA is the major immunoglobulin and IgA deficient individuals experience higher rates of respiratory infections[90,112].

Lastly, regulatory T-cells (Tregs) are tasked with resolution of inflammation upon infection clearance. Their job is crucial in collateral damage control caused by pro-inflammatory immune mechanisms. Originating either from the thymus (natural Tregs) or from conventional CD4+ T-cells differentiated in the periphery (induced Tregs), these cells are potent immune suppressors. They downscale immune activation, kill effector T-cells, limit growth factor availability, and promote tissue repair returning the lung back to homeostasis[113-115].

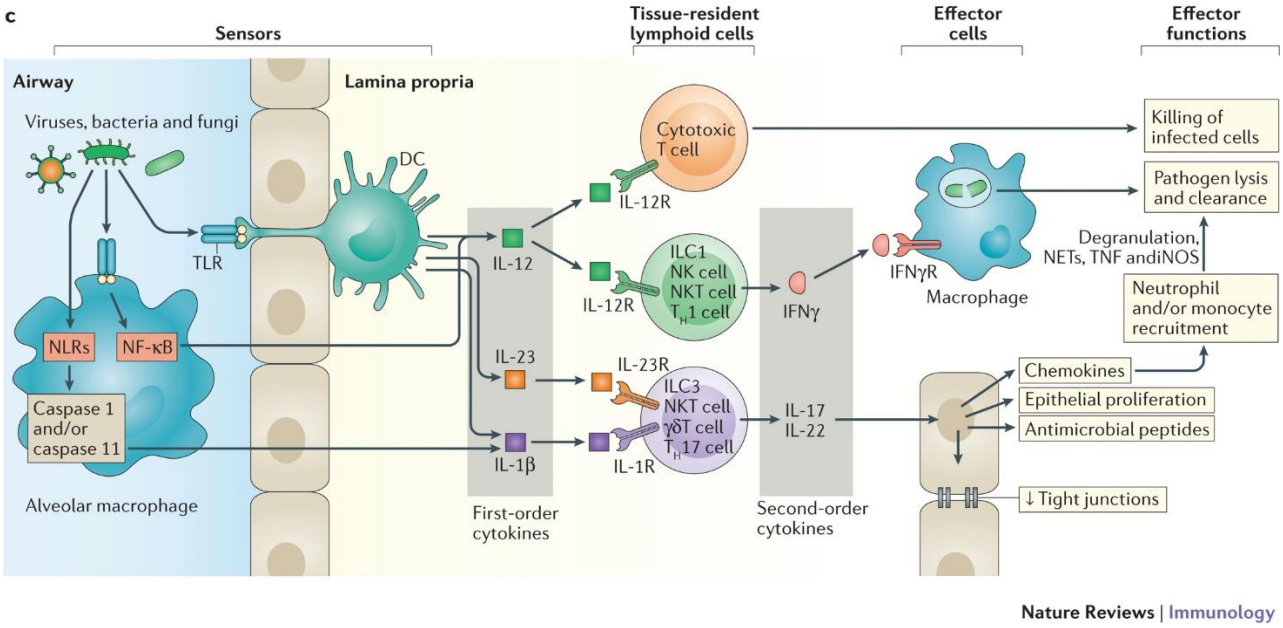

Figure 10: Tiers of the early Type I lung immune response to infectious agents. Toll-like and NOD-like receptors (TLRs and NLRs) expressed by AMs and DCs help them bind and recognize viral, fungal, and bacterial molecules in the airway. Recognition of these molecules when inhibitory signals are disrupted triggers first-order cytokine production, engaging the adaptive branch of the immune system. Adaptive immune cells further enhance direct killing of the pathogen or infected cells and recruit more effector cells from the circulation[95].

CHAPTER 3: Pulmonary Immune Dysregulation During HIV infection

3.1 Acute HIV and Lung Immunity:

Within a few hours following transmission, HIV begins to replicate in mucosal, submucosal, and draining lymphoid tissues. Notably, lung is an early target of HIV dissemination because it is highly vascularized and houses a very large pool of target cells. Experimental SIV-infections of macaques demonstrate that the virus is seeded into the lungs shortly after intravenous infection[116-118]. In fact, SIV replication in BAL cells of pigtailed macaques can be detected as early as 7 days post-inoculation, peaking at 10 days during acute infection[117]. Interestingly, the CCR5-tropic HIV strain, known as HIV-1Bal, was originally isolated from the lungs[119]. Viral quasispecies specific to this tissue and distinct from the circulation have also been reported[120,121].

Upon reaching the lung, HIV is seeded into multiple cell types: CD4 T-cells, DN T-cells, AMs, and bronchial epithelial cells. Although HIV preferentially infects CD4 T-cells, which account for ~6% of total BAL cells in healthy non-smokers, AMs, which account for ~85% of total BAL cells, are also infected[98,122-124]. Although data on pulmonary immune perturbations during primary acute HIV infection is scarce, *in vitro* experiments on human lung lymphocytes and *in vivo* animal SIV models suggest that during the acute phase of infection pulmonary interstitial CD4 T-cells are more severely and rapidly depleted compared to the blood compartment that is largely due to CCR5+ memory CD4 T-cells' high susceptibility to CCR5-topic HIV-1 infection, which make up the vast majority of the lung CD4 T-cell pool[125,126].

Most acute HIV-1 infections are caused by the R5-tropic strain (M (Macrophage)-tropic strain), which targets both T-cells and macrophages. Although this strain is not very efficient at infecting macrophages on its own, its infectivity is enhanced significantly during cell-to-cell contact between AMs and infected CD4 T-cells, as has been recently shown by Schiff and his group, suggesting that AM HIV entry during acute infection is CD4 T-cell dependent[124]. Within the AM cell pool (CD206+) two subsets have been identified based on their size and granularity– small and large AMs[127]. Human *ex vivo* AM analysis shows that HIV preferentially

infects monocyte-like small AMs, which show higher pro-inflammatory gene expression and greater phagocytic capacity compared to large AMs[127,128]. In addition to infecting AMs, we have also shown in a humanized mouse model of early HIV infection that the virus is preferentially seeded within lung DN T-cells, a rather novel lung HIV reservoir cell subset, which is enriched in the lungs of both HIV+ and seronegative individuals compared to other tissues[129]. Lastly, some groups have shown that HIV-1 can productively infect bronchial epithelial cells *in vitro*. Devadoss and colleagues have demonstrated that normal human bronchial epithelial cells grown on air–liquid interface can be infected by X4-tropic HIV-1 in a CXCR4/CD4-dependent manner resulting in production of p24, a marker of productive HIV-infection. Moreover, they have shown that lung epithelial cells from HIV-infected humans and simian-adapted HIV-infected macaques contained HIV RNA transcripts, suggesting that these cells could act as potential HIV reservoirs[130].

In about two thirds of cases of primary HIV infection, individuals experience flu-like symptoms such as fever, chills, and swollen lymph nodes. Some individuals also suffer from respiratory symptoms such as a sore throat or a dry cough, suggesting engagement of the pulmonary innate immune response[131]. Massive CD4 T-cell apoptosis during the acute infection phase is accompanied by profound immune activation, caused by release of apoptotic microparticles into the bloodstream[43,132]. Furthermore, in addition to the attack on human immune cells, HIV also compromises the integrity of the lung epithelial barrier by infecting human bronchial epithelial cells, decreasing their expression of E-cadherin, and increasing paracellular permeability[133,134].

3.2 Untreated Chronic HIV and the Lung Immunity:

In chronic HIV infection, viral reservoirs in deep tissues are already well established, including those in the lung tissue. In fact, SIV-models show that, during the asymptomatic phase of infection, there is no correlation between plasma and BAL fluid viral loads[117]. This is further supported by human phylogenetic studies, which demonstrate HIV lung reservoir compartmentalization in untreated patients[135,136]. Furthermore, whole lung tissue biopsies of untreated HIV-infected persons harbor distinct viral quasispecies compared to those found in

their blood and lymphoid tissues suggesting that the HIV reservoir may be replicating and evolving locally in that anatomical site, rather than solely spreading from the circulation[120,121].

Prior to the introduction of ART, lung infections were the leading cause of death in people living with HIV (PLWH)[137]. These were largely caused by *Pneumocystis jirovecii pneumoniae* (PCP) in developed countries and *Mycobaterium tuberculosis* in underdeveloped countries[138,139]. As seen in the very first AIDS reports in gay men in the 1980s, PCP infections—which were previously rare fungal pulmonary infections became a staple AIDS-defining illness observed in severely immunosuppressed patients[140,141]. Untreated PLWH are also at a higher risk of developing recurring bacterial pneumonia, whose rate is inversely proportional to the patients' CD4 T-cell counts[142]. They are also more prone to cancer development, such as lung cancer, compared to persons without HIV infection[143,144].

The dysregulated pulmonary immune environment in PLWH may facilitate the development of such lung pathologies. Destruction of lung parenchyma, pulmonary inflammation, and emphysema are recognized complications of HIV infection. Up to 60% of untreated PLWH present with lymphocytic alveolitis, characterized by infiltration of B-cells, gamma-delta T-cells, as well as CD4+ and CD8+ T-cells into the lung, which is observed in absence of any respiratory symptoms[145-148]. Both, their pulmonary CD8 and CD4 T-cells show 2- to 3-fold greater expression of HLA-DR and CD38, which are markers of immune activation, compared to seronegative adults[149]. In untreated PLWH with lymphocytic alveolitis, Neff and colleagues have shown lung HIV-specific CD4+ and CD8+ T-cells exhibit impaired proliferative capacity, which is caused by high expression levels of PD-1, a classic exhaustion marker that dampens T-cell receptor signaling[145,150]. Furthermore, the lung cytokine milieu of PLWH is disrupted. Jambo *et al* have shown that BAL fluid taken from ART-naïve HIV+ study participants have increased concentrations of RANTES and TNF-β and a shift towards MIP-1β, MCP-1, and IP-10 signaling network[151]. Notably, RANTES is a lymphocyte chemoattractant, shown to a play a role in lung CD8 T-cell recruitment in other viral lung infections. These CD8 T-cells can, in turn, produce TNF-β, a potent pro-inflammatory cytokine that promotes vascular cell adhesion, chemokine production, and further immune cell infiltration into the tissue[152-154]. IL-6 is another pro-inflammatory player, whose levels are associated with higher HIV RNA levels and has

repeatedly been shown to be produced by monocytes and macrophages in response to HIV[155,156]. *In vivo* non-human-primate models further show that IL-6 expression in pulmonary interstitial macrophages of SIV-infected animals is positively correlated with monocyte turnover and lung tissue damage[157]. As mentioned previously, HIV has also been shown to impair pulmonary epithelial integrity by decreasing expression of cell-to-cell adhesion molecules and promoting further release of pro-inflammatory mediators by these cells, thus accelerating the decline in lung function[133].

Chronic pulmonary inflammation further leads to increased production of matrix metalloproteinases (MMPs), a family of endopeptidases that can degrade elastin and collagen fibers[158]. Notably, both elastin and collagen degradation products act as immune cell chemoattractants: while elastin fragments recruit monocytes, collagen fragments attract neutrophils[159]. Collectively, MMPs play a role in tissue repair and modulate the immune response. *In vitro* experiments on primary human airway basal cells have shown that HIV infection can force these cells to acquire a destructive phenotype via upregulation of MMP-9 through activation of MAPK signaling, thus potentially contributing to emphysema development in PLWH[160].

On the other end of the scale between inflammation and wound repair, HIV leads to higher levels of TGF-β – an anti-inflammatory cytokine produced by Tregs and alveolar macrophages. Because PLWH experience persistent low-grade chronic inflammation, that is in part caused by bacterial translocation across the gastrointestinal mucosal barrier, the immune system tries to counteract it via anti-inflammatory cytokines, such as TGF-β[161,162]. Notably, TGF-β levels are significantly higher in PLWH compared to seronegative individuals and remain elevated regardless of ART treatment and viral load suppression[163]. TGF-β can downregulate inflammatory processes by promoting Treg expansion and inhibiting effector T-cell function, as well as drive collagen deposition by fibroblasts as part of the normal wound repair process[164,165]. In PLWH, long-term TGF-β elevation may contribute to irreversible tissue fibrosis of the gut, secondary lymphoid organs, and the lung[166-169]. One study has also shown higher TGF-β production by AMs from PLWH compared to AMs from healthy donors, which the authors believe to be implicated in impaired IgG secretion in the alveoli[170]. AMs from PLWH

also show a pro-inflammatory phenotype, higher TNFα production, and impaired phagocytic ability, which in turn leads to poor pathogen clearance[128,171-173]. Untreated HIV infection also leads to loss of anti-inflammatory CD163+CD206+ AMs[174]. Furthermore, AMs from SIV infected macaques show elevated levels of PD-1 which positively correlates with plasma viral load, suggesting that higher PD-1 expression on AMs may be associated with disease progression[175].

3.3 ART-treated HIV and lung immunity:

Although introduction of ART has greatly reduced the rate of opportunistic infections and improved the quality of life of PLWH, it does not fully restore all immune perturbations caused by HIV infection. Instead, the spectrum of the most prevalent pulmonary diseases had shifted from opportunistic infections to chronic illnesses, such as emphysema, chronic obstructive pulmonary disease (COPD), pulmonary fibrosis, and lung cancer which are discussed in more detail in a later section. Notably, although ART had greatly reduced the rate of lung infections in PLWH, they still occur more frequently in HIV infected individuals compared to the general population[176,177].

Viral control after ART-initiation has a significant impact on lung immunity. Lymphocytic alveolitis resolves, CD8 T-cell numbers decrease, CD4 T-cell count improves, and thus CD4:CD8 T-cell ratio is ameliorated[178,179]. Notably, lung CD4 T-cell repopulation likely occurs due to local expansion of the tissue resident subset, as seen by higher Ki67 expression in BAL CD4 T-cells 1 month after starting therapy[179]. Twigg *et al* have also demonstrated that level of CD38 and HLA-DR on BAL lymphocytes decrease significantly after the first 6 months of therapy, especially on CD8+ T-cells[180]. However, as our team has recently demonstrated, levels of HLA-DR+CD38+ BAL CD4 T-cells remain higher in ART-treated PLWH compared to healthy controls[60].

Twigg and his group also show that intracellular levels of INF-γ, TNF-β, and IL-2 in alveolar CD4+ and CD8+ T-cells, measured after mitogenic stimulation with the superantigen staphylococcal enterotoxin B, decline significantly in ART-treated PLWH[180]. Similar reports were made on extracellular levels of inflammatory cytokines in BAL fluid, such as INF-γ, IL-6, and INF-γ inducible chemokines like IP-10[180-182]. In contrast, Knox *et al*, showed that although BAL CD4 T-cell infection rate decreases with introduction of ART, both CD4 and CD8 T-cell polyfunctional

profiles (INF-γ, TNF-α, IL-2) after mitogenic stimulation remain relatively unchanged[179]. Importantly, this was not observed in the peripheral blood, where CD8 T-cells showed a marked improvement in polyfunctional cytokine secretion in response to a stimulus[179]. These findings further highlight that immune restoration in lung tissue during ART is incomplete, which can help explain ongoing susceptibility of treated PLWH to pulmonary infections[183,184].

Apart from pro-inflammatory mechanisms, anti-inflammatory immune functions also remain dysregulated during ART. PLWH have higher levels of CD39+CD73+Tregs in their BAL fluid compared to peripheral blood, while no such difference is observed in seronegative controls[60]. Importantly, these immunosuppressive cells can act as a double-edged sword. While they can help resolve inflammation during acute lung injury, they are also capable of promoting tumor cell survival, angiogenesis, and fibrosis[185,186].

AM dysfunction is yet another factor contributing to compromised lung immunity in PLWH on ART. Collini *et al* have shown that AMs from ART-treated PLWH have defects in microbicidal mechanisms, mediate by HIV's gp120 protein, which could inhibit macrophage apoptosis induction, caspase activation, and mROS-dependent pneumococcal killing[187]. These cells also remain subjected to chronic oxidative stress despite ART. Yeligar *et al* have shown that BAL fluid from treated PLWH has higher H_2O_2 concentration[188]. Furthermore, their AMs have lower expression of proliferator-activated receptor (PPAR)-γ, an important player in combatting oxidative stress during acute lung injury, higher expression of NADPH oxidases, which further promote oxidative stress and inhibit phagocytosis, and higher levels of TGF-β, a big driver of tissue fibrosis[188-191]. Impaired AM phagocytic capacity and skewing in polarization has been further emphasized by Akata *et al*[192]. Using BAL samples from PLWH and healthy controls, they have demonstrated that, out of the four macrophage subsets (non-polarized: CD40-, CD163-; M1: CD40+, CD163-; M2: CD40-, CD163+; double-polarized: CD40+, CD163+) the double-polarized subset has the highest phagocytic capacity. Notably, this subset was significantly diminished in HIV+ COPD- individuals, while the non-polarized subset, which had the lowest phagocytic capacity, was enriched[192]. The collective effects of HIV on pulmonary inflammation are summarized in Figure 12.

3.4 ART toxicity:

Interestingly, there are several studies which suggest that ART toxicity might also play a role in immune dysregulation in PLWH. *In vitro* studies have shown that nucleoside reverse transcriptase inhibitors can decrease mitochondrial DNA content and complement-mediated phagocytosis in human monocyte-derived macrophages[193]. In addition, Korencak *et al* looked at CD4 T-cells from treated and untreated PLWH, as well as HIV-uninfected individuals, and showed that ART improves these cells' metabolic phenotype but not the respiratory impairment, especially in patients receiving integrase inhibitors. Furthermore, they show that CD4 T-cells treated with dolutegravir and elvitegravir shift their cytokine response from a polyfunctional response to a TNF-α dominated one[194]. A similar observation was made by Bowman and colleagues, who demonstrated that human macrophages exposed to tenofovir disoproxil fumarate and emtricitabine have lower mitochondrial mass and increased lipid uptake[195]. These studies can help explain the observations made by Correa-Macedo *et al* showing the potential adverse effect of ART on transcriptional response of AMs to *Mycobacterium tuberculosis* (*Mtb*). In their recent study, they examined isolated AMs from healthy controls, PLWH on ART, as well as seronegative participants taking pre-exposure prophylaxis (PrEP). Notably, AMs from HIV+ and HIV- PrEP study groups both had a weaker transcriptional response when challenged with *Mtb* compared to AMs from HIV uninfected controls. Furthermore, AMs from HIV- PrEP and HIV+ donors showed no change in chromatin state upon challenge, unlike AMs from healthy controls which displayed a significant change in chromatin accessibility[196]. Collectively, although ART greatly improves the quality of life of PLWH and restores many immune parameters, these findings suggest that ART is not innocuous. Some regimens may decrease mitochondrial function and elicit a pro-inflammatory immune cell profile, thus partially contributing to chronic inflammation in treated individuals.

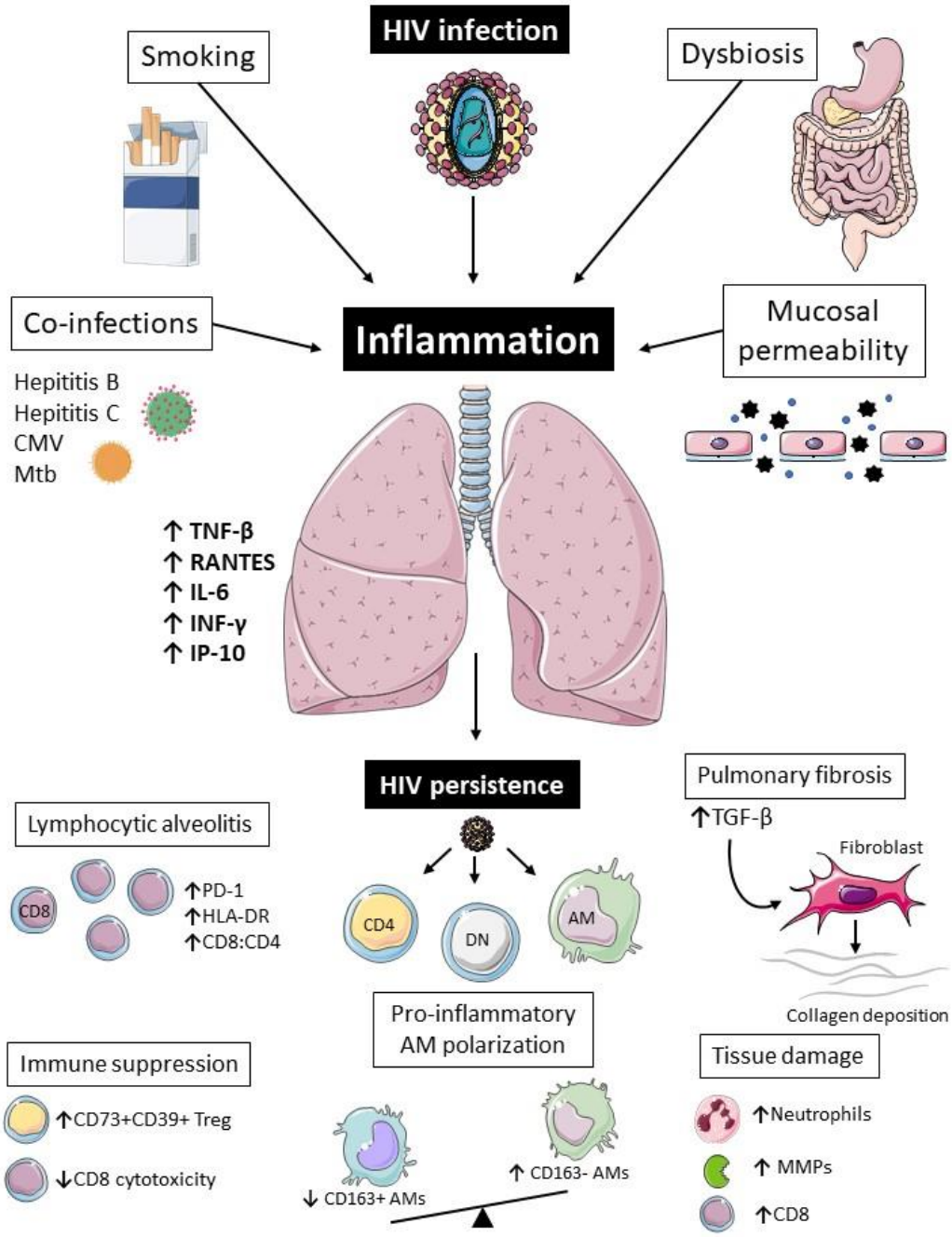

Figure 12: Inflammation is likely the biggest driver of pulmonary pathologies and lung HIV reservoir persistence in PLWH. Apart from the virus itself, which seeds the lung within the first few weeks of infection, other factors also contribute to pulmonary immune perturbations in PLWH during ART, such as smoking, co-infections, changes in the microbiome, and compromised integrity of mucosal barriers. Collectively, these factors fuel chronic inflammation and pulmonary immune activation characterized by

high levels of pro-inflammatory cytokines (RANTES, TNF-β, IFN-γ, IL-6, IP-10), which in turn lead to immune cell recruitment to the lung tissue, typically presenting as lymphocytic alveolitis. These CD8 T-cells appear as functionally impaired and fail to remove lung HIV reservoir that continues to persist in mucosal CD4+ T-cells and CD4-CD8- DN T-cells, as well as alveolar macrophages (AMs). A vicious cycle of immune activation and residual viral replication ensues and subsequent pulmonary immune abnormalities, such as pro-inflammatory AM polarization, extracellular matrix destruction caused by increased production of matrix metalloproteinases (MMPs) and increased neutrophil count. In an attempt to counteract this inflammatory process, immunoregulatory arm of the immune system could further contribute to increased risk of pulmonary co-morbidities, such as accumulation of immunosuppressive regulatory T-cells (CD73+CD39+Treg) and higher levels of TGF-β[197].

CHAPTER 4: Pulmonary Immune Dysregulation During Smoking

4.1 Tobacco Smoke Toxicity

Cigarette smoke consists of over 4500 chemical compounds, which include carcinogens (methylcholanthrene, benzo-α-pyrenes, acrolein), toxins (carbon monoxide, ammonia, acetone, nicotine, hydroquinone), and oxidants (superoxides, nitrogen oxides)[198,199]. These constituents can cause tissue damage by increasing lung oxidant burden and by upregulating pro-inflammatory immune mechanisms. Increased oxidant burden has been well documented in smokers and COPD patients, which subsequently leads to generation of oxidative moieties and DNA adducts[200,201]. For instance, levels of 4-Hydroxy-2-Nonenal (4-HNE), a highly reactive and diffusible lipid peroxidation product, is elevated in lungs of COPD patients. Notably, 4-HNE has been shown to cause cellular and mitochondrial dysfunction in human small airway epithelial cells and induce cellular stress responses[202,203].

Cigarette-smoke induced oxidative stress has been implicated in multiple inflammatory lung diseases, including asthma, COPD, and pulmonary fibrosis[204,205]. Notably, reactive oxygen species (ROS) contributing to this stress are derived not only from inhaled tobacco smoke but also from activated immune cells like macrophages, epithelial cells, neutrophils, and T-cells, which produce ROS as an antimicrobial mechanism, capable of causing direct damage to DNA, lipids, and proteins[206-208]. Moreover, smoking not only contributes to oxidative stress but also compromises the lung's antioxidant defense system. Avti and colleagues have reported that long-term use of aqueous extract of gutkha (a form of smokeless tobacco) reduces glutathione levels, an important antioxidant, and inhibits the activity of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, likely due to an excessive amount of free radicals that are generated[209]. Importantly, glutathione is not only crucial for effective detoxification of ROS and free radicals in the pulmonary milieu. In fact, Diotallevi *et al* have demonstrated that glutathione is implicated in fine tuning of the antiviral immune response in macrophages independently of its antioxidant properties[210].

4.2 Effects of Smoking on Lung Immune System

Pulmonary epithelium:

It is well known that cigarette smoke compromises the function and integrity of the pulmonary epithelial cell barrier. Cigarette smoke extract has been shown to disrupt cell-to-cell contacts likely via epidermal growth factor receptor activation and subsequent protease calpain mediated degradation of tight junctions[211]. Furthermore, smoking alters basal cell differentiation and leads to pathological remodeling of the small airway epithelium[212]. As a consequence, smoking results in increased paracellular permeability, mucous overproduction, and impaired mucociliary clearance of the lung mucosa[213,214]. Curiously, although cigarette smoke is known to trigger a pro-inflammatory response in human primary bronchial epithelial cells, it was also reported that it can attenuate the production of some inflammatory mediators, such as those involved in Type I Interferon-mediated antiviral immunity, following stimulation with lipopolysaccharides or double stranded RNA[215-218].

Cytokine production and immune cell function:

As alluded to previously, cigarette smoke can have both pro- and anti-inflammatory effects. It has been shown to induce production of TNF-α, IL-1, IL-6, IL-8, and monocyte chemoattractant protein-1[219,220]. However, other studies indicate that nicotine, the most addictive component of tobacco, does the opposite, where it can decrease levels of IL-6, IL-8, and IL-10 through the engagement of α7 nicotinic acetylcholine receptor on macrophages, T-cells, and B-cells[221-223].

Increased production of pro-inflammatory cytokines induced by cigarette smoke has a significant effect on BAL fluid immune cell concentration and composition. Specifically, bronchoalveolar lavage (BAL) fluid from smokers has increased number of neutrophils, macrophages and CD8 T-cells compared to non-smokers[224,225]. Furthermore, due to increased CD8 T-cell frequencies, CD4/CD8 T-cell ratio in BALF of smokers is reduced. Studies also show that these CD8 and CD4 T-cells are skewed towards a type 1 phenotype (Tc1 and Th1 cells respectively), which are IFN-γ producing cells that help fight intracellular infections but are also capable of causing tissue destruction in COPD patients[226-228]. Skewed T-cell polarization in smokers could be partially caused by afore mentioned neutrophilic inflammation. Because

cigarette smoke was shown to be able to trigger release of neutrophil extracellular traps (NETs), an important mechanism of innate immune defense, it is hypothesized that these NETs can drive plasmacytoid dendritic cells maturation, which promote Th1 and Th17 CD4 T-cell responses[229].

Additionally, cigarette smoke exposure has been associated with increased B-cell deposition in the lung, further supporting the notion that smoking induces an adaptive immune response. Brandsma and colleagues have reported both a higher percentage of total B-cells in lung tissue as well as increased levels of IgG- positive memory B-cells in current smokers compared to ex- and never-smokers[230].

Smoking also affects AM polarization. However, observations made on AMs from BAL and from whole lung tissue differ. Immunohistochemistry analysis of lung tissue taken from smokers with normal lung function and non-smoking healthy controls has revealed that there is an increase in pro-inflammatory M1 AMs in small airway epithelium[231]. Similarly, other groups studying surgical lungs report that the percentage of both M1 and M2 AMs increase progressively with smoking and COPD severity[232].

In contrast, studies performed on BAL samples demonstrate that luminal AMs taken from current smokers show significantly higher frequency of non-polarized cells (neither M1 nor M2) compared to ex-smokers, which is the same phenomenon that has been observed in PLWH[233]. Older reports looking at gene expression of BAL AMs from smokers and non-smokers with normal lung functions also indicate a downregulation in M1- and upregulation of M2-associated genes[234].

Despite the seemingly contradictory observations regarding AM polarization due to interstudy differences and their respective definitions of M1/M2 phenotypes, one thing remains clear – smoking impairs AM phagocytic capacity and antimicrobial function, as has been shown in both *in vitro* an animal models[235-237].

4.3 Smoking and respiratory infections

Epidemiological studies have repeatedly shown that cigarette smoking is associated with higher rates of viral and bacterial respiratory infections. Smokers are at a 2- to 4-fold increased risk of invasive pneumococcal disease[238,239]. Smokers are also more susceptible to the common cold and the flu compared to non-smokers[240]. Importantly, the attributable risk for severe influenza in smokers was reported to be around 40%[241,242]. Other increased infection risks in smokers *versus* non-smokers include varicella pneumonitis (15-fold higher), tuberculosis (2-fold higher), and Middle East respiratory syndrome coronavirus illness (7-fold higher)[243-245].

During the ongoing COVID-19 pandemic, some studies have documented an inverse relationship between smoking and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. This in turn led to a lot of confusion in the medical community and the general public, suggesting that smoking could protect against SARS-CoV2 infections[246-248]. Fortunately, these false and rather dangerous assumptions are slowly being dispelled. Westen-Lagerweij *et al* have recently published an excellent summary elucidating the origins of this myth[249]. They explain that smokers were significantly underrepresented among COVID-19 patients recruited in these studies. Moreover, the authors made their comparisons without adjusting for other confounding variables like age, gender, socio-economic status, ethnicity and occupation. This myth is also further being disproven by new emerging studies, which show that both hospitalization and mortality risks due to SARS-CoV2 infection are higher among smokers than in never smokers[250,251].

CHAPTER 5: Accelerated pulmonary co-morbidities during HIV infection

5.1 Accelerated Immune Aging:

Many age-related co-morbidities occur in PLWH at a younger age than in the general population. This premature onset of age-related illnesses has been largely attributed to chronic inflammation and immune activation, which in turn leads to accelerated immune aging, now known as 'inflammageing'[80,252]. Inflammageing is characterized by high level of circulating pro-inflammatory cytokines (IL-6, IL-8, TNF-α, and IFN-γ), telomere shortening, cell senescence (loss of CD28 and increased expression of CD57 and KLRG1 on T-cells), mitochondria dysfunction, and changes in microbial composition of the microbiota, all of which have been documented in PLWH[252-255]. Consequently, this accelerated immune aging affects the lungs. Leung *et al* had conducted several studies on telomere shortening in PLWH[256,257]. They have shown that telomeres in circulating leukocytes of PLWH are significantly shorter than in seronegative study participants. Furthermore, their telomere length correlated with severity of poor lung function determined by forced expiratory volume[256]. Notably, in their more recent publication, which included HIV+ participants with good immune recovery and undetectable viral loads, the telomere length of their small airway epithelial cells was significantly shorter than in healthy controls, even after accounting for cigarette smoke exposure[257]. Moreover, HIV+ participants in that study were on average 4 years younger than the seronegative ones.

5.2 HIV and COPD:

COPD is a progressive inflammatory lung condition characterized by airway obstruction, inflamed mucous membranes and alveolar damage and is the third leading causes of death worldwide with 2 million people affected in Canada alone[258,259]. Both smoking and HIV are independent risk factors for COPD development[260-262]. Furthermore, COPD has often been proposed to be a disease caused by accelerated immune aging[263,264]. A recently published study conducted by Córdoba-Lanús *et al* monitored telomere length of patients with COPD over a 10 year period and found an association between accelerated telomere shortening and

progressive decline in lung function, such as worsening of gas exchange and lung hyperinflation in COPD patients[265].

HIV-associated COPD also further dysregulates lung immune cell function. Popescu *et al* have documented that HIV+COPD+ individuals show severe CD4 T-cell loss in their BAL fluid, mediated by Fas-dependent activation-induced cell death. Their BAL CD4 T-cells also show poor HIV-specific immune response and loss of polyfunctionality compared to HIV+COPD− participants[266]. Curiously, unlike lung mucosal CD4 T-cells, BAL CD8 T-cells in these individuals maintained their HIV-specific function. Moreover, increased CD8 T-cell cytotoxicity has been documented previously in COPD patients, which might contribute to this condition's highly tissue destructive phenotype[267].

5.3 HIV and Lung Cancer:

Lung cancer is a leading cause of death, and especially cancer-related death, in PLWH[268]. Notably, HIV and COPD are both risk factors for lung cancer development even after accounting for smoking status[269-271]. Increased lung cancer risk in PLWH has been attributed to acute inflammatory insults caused by lung infections, chronic low-grade inflammation, CD8 T-cell dysregulation, compromised integrity of pulmonary epithelium, and changes in lung microbiome[272-275]. Accelerated immune aging may also play a role. Klugman and colleagues have shown that in the United States PLWH were diagnosed with non-small cell lung cancer at a younger age compared to seronegative participants, with lower median survival time especially among those with a low CD4/CD8 ratio and high viral loads[276]. Exhausted CD8 T-cells likely contribute to worse lung cancer outcomes in PLWH, especially in those with multiple co-infections which exacerbate this exhausted phenotype further[277,278]. Given the importance of these cells in fighting cancer, their exhaustion leads to decreased responsiveness to stimuli, low cytotoxicity, poor IFN-γ secretion, and thus compromised ability to kill tumor cells[279-281].

5.4 HIV and Pulmonary Fibrosis:

The high rates of pulmonary fibrosis in PLWH compared to those in the general population could be attributed to increased TGF-β levels[169,282]. TGF-β levels are significantly higher in PLWH compared to seronegative individuals and remain elevated regardless of ART treatment and

viral load suppression[163]. Because TGF-β can drive collagen deposition by fibroblasts it has been implicated in irreversible tissue fibrosis of the gut, secondary lymphoid organs, and the lung in PLWH[166-169]. A 2017 multi-center Lung-HIV study has shown that fibrotic lung changes have been observed in 29% of HIV-infected participants, which correlated with viral load but not ART treatment status or CD4 T-cell count [169]. Of note, high levels of TGF-β in PLWH might contribute to poor non-small lung cancer outcomes, as it has already been documented in seronegative individuals, although a direct link between TGF-β levels in PLWH and lung cancer outcomes is yet to be established[170,283-285].

5.5 HIV and Pulmonary Emphysema:

In contrast to pulmonary fibrosis, emphysema is characterized by higher lung compliance, increased lung volume and lower expiratory flow rate[286]. Increased risk of PLWH of pulmonary emphysema was reported as early as the 1980s[287]. More recent studies further confirmed that HIV status is a risk factor for emphysema development independently of smoking[288,289]. Some of the mechanisms in PLWH that likely contribute to this risk have been mentioned previously. These include CD8 T-cell accumulation, increased oxidative stress, AM activation, and increased production of MMPs caused by chronic lung inflammatory state, which can subsequently lead to extracellular matrix destruction[159,160,289]. Attia *et al* have further demonstrated that participants in their HIV+ study group were more likely to have a greater portion of their lung to be affected and were more likely to be diagnosed with COPD compared to seronegative controls diagnosed with emphysema. Additionally, in their HIV+ study arm, low CD4 T-cell counts and high soluble CD14 levels were linked with disease severity, supporting the notion that immune activation in PLWH contributes to the risk of pulmonary emphysema development[288]. Whether ART initiation decreases the risk of emphysema development remains rather unclear. Emphysema rates in the pre-ART era were reported to be 15% in PLWH *versus* 2% in the general population[290]. In ART-treated patients, the reported incidence rates are even higher. Guaraldi *et al* have reported that, of 1,446 HIV-infected patients on ART in their cohort, nearly 50% had evidence for emphysema and/or bronchiolitis based on thoracic computed tomography (CT) scans, with 13% showing signs of bronchiolitis, 19% showing emphysema, and 16% having both[291]. Furthermore, among ART-treated HIV-infected

participants recruited by Leung et al., emphysema progression was not associated with peripheral CD4 cell counts or CD4:CD8 ratio, HIV viral load, ART classes or duration of ART exposure[292]. As proposed by others, the increase in incidence rate of chronic inflammatory conditions in the ART era, of not just emphysema and COPD but also diabetes and cardiovascular disease, can largely be explained by improved life expectancies in treated PLWH, giving them more time to develop these co-morbidities[293,294].

5.6 Role of Smoking in Pulmonary Co-morbidities:

In a nationwide population-based cohort study conducted, Helleberg and others reported that both all-cause and non-AIDS-related mortalities are higher among HIV+ smokers compared with HIV+ non-smokers. They also highlight that smoking PLWH lose more life-years to smoking than to HIV itself (12.3 years versus 5.1 years respectively)[295]. Furthermore, they show that smoking-associated risk of death was 61% among PLWH compared to 34% among healthy controls. Notably, risk of death among former smokers was reduced by 40% compared to current smokers. Around 70% of myocardial infarctions and 27% of cancers in PLWH are related to their smoking status[296]. Importantly, unlike with myocardial infarction risk, cancer risk could remain elevated in former smokers even 5 years after smoking cessation[296].

Smoking further increases the risk of COPD and emphysema in PLWH[289,297]. Notably, pulmonary emphysema is more prevalent in HIV+ smokers compared to seronegative smokers and is often developed at a younger age, which could be partially attributed to immune dysregulation of AMs in PLWH[289]. Indeed, previous *ex vivo* human studies show an increase in MMP expression in both AMs and epithelial lining fluid in HIV positive smokers with early emphysema compared to HIV negative smokers with the same lung condition [298]. Previous reports also show that smoking can activate cytotoxic CD8 T-cells, which can in turn exacerbate pulmonary injury[299-301].

CHAPTER 6: Lungs as an HIV Reservoir

6.1 Lungs Provide Ideal Grounds for HIV Spread:

As organs, the lungs possess several features that may contribute to HIV reservoir establishment, several of which stem from their anatomy. Similarly to the gastrointestinal tract, a confirmed and well-studied HIV reservoir site[302], the lungs are an extension of the external environment. They are constantly exposed to external particles and airborne microorganisms. The antigen load in the lungs is therefore quite high. Furthermore, although lymphocytes make up only 10% of the BAL cell pool, the lymphocyte count in pulmonary interstitium is comparable to that of peripheral blood, with as many as 10 X 10^9 cells[83]. High antigen load can in turn promote activation of these lymphocytes and other immune cells, consequently supporting HIV replication and continuous reservoir replenishment in the lung.

Because the lungs carry out the vital function of gas exchange, they are highly vascularized. Their high blood flow, cell proximity, surface area, and small arteriole size could further aid in HIV cell to cell spread and reservoir compartmentalization[83]. Furthermore, the lungs of HIV-infected persons harbor distinct viral quasispecies that are tissue specific suggesting that HIV reservoir may be replicating locally in that anatomical site, rather than spreading from other infected tissues[120,121].

6.2 HIV Persists in Multiple Lung Immune Cell Types:

Early viral presence in the lung, along with high rate of co-morbidities caused by lung diseases in PLWH even after ART initiation, support the notion that lungs serve as another anatomical HIV reservoir. Our team previously assessed HIV persistence in the pulmonary milieu in individuals under long-term treatment (median 9 years)[303]. We found that total HIV DNA in BAL CD4 T-cells was significantly higher than in peripheral blood mononuclear cells. Moreover, the lungs were enriched in activated memory CD4+ T-cells subsets that can further promote HIV replication and persistence [67]. We also observed that pulmonary mucosal DN T cells of PLWH on ART expressed higher levels of HLA-DR and several cellular markers associated with HIV persistence (CCR6, CXCR3, and PD-1) compared to the blood [129]. Importantly, CD3+CD4-CD8-

DN T-cells from the BAL fluid of these participants harbored HIV DNA. Using the humanized bone marrow-liver-thymus mouse model, our group also observed higher infection frequencies of lung DN T-cells than those of the blood and spleen in both early and late HIV infection stages, meaning that apart from AMs and CD4 T-cells, HIV is also seeded in pulmonary mucosal DN T-cells early following infection and persists in these potential cellular HIV reservoirs even during long-term ART [129].

AMs pose yet a bigger challenge on our way to HIV reservoir eradication due to their abundance, longevity, and resistance to apoptosis[187,304]. As alluded to previously, HIV viral proteins remain detectable in BAL fluid of treated HIV-1 infected patients, as Collini and his group have demonstrated, which further underscores the role that AMs serve as HIV cellular reservoirs in the lung despite ART [187]. Clayton and colleagues also show that HIV-infected macrophages are resistant to CD8 T-cell mediated killing, even more so than HIV-infected CD4 T-cells, which is associated with increased pro-inflammatory cytokine production[305]. The relative importance of the lungs as viral reservoirs has also been highlighted by Horiike *et al* in an SIV-infected Rhesus macaque model, where they found that the lungs and intestines of ART-treated animals had the largest burdens of SIV RNA, second to the lymphatic tissues[306]. HIV persistence in the lung during ART has been further confirmed by Santangelo's group using antibody-targeted positron emission tomography – a real-time, *in vivo* viral imaging method, showing that although lung viral signals are reduced after ART initiation they still remain detectable[307].

CHAPTER 7: Tissue-resident CD8 T-cells

7.1 CD8 T-cell origin and development

CD8 T-cells are key players of the adaptive immune system in the body's defense against intracellular infections and cancers. These cells develop from the bone marrow's lymphoid progenitors that migrate to the thymus, where thymic epithelial cells instruct them to fully commit to the T-cell lineage via Notch signaling, which initiates T-cell specific gene expression[308]. These developing T-cells, termed double negative thymocytes, do not yet express neither the CD4 nor the CD8 co-receptors. After extensive proliferation and successful α- and β-chain locus rearrangement of the T-cell receptor (TCR), they begin to express both CD4 and CD8 and undergo a rigorous selection process. Cells that don't recognize a self-peptide:self-MHC molecular complex will die by neglect during "positive selection". Those that do then cease to express either the CD4 or the CD8 co-receptor. These single positive thymocytes undergo a negative selection process, which eliminates any cells that bind too strongly to ubiquitous self-antigens to avoid autoimmune pathology. Only a tiny fraction of the double positive thymocytes survive this dual screening process, giving rise to naïve CD4 and CD8 T-cells[309].

Naïve CD8 T-cells continuously recirculate between the blood and secondary lymphoid organs. To home to a lymph node, naïve T-cells express CCR7, a chemokine receptor, and CD62L, an L-selectin, that allow them to bind and traffic across the high endothelial venules (HEV) into the T-cell zone. While CD62L binds to Peripheral node addressin (PNAd) on HEV to begin tethering and rolling, CCR7 binds to CCL21 and CCL19 secreted by the T-cell zone, which mediate integrin activation, rolling arrest, and subsequent extravasation[310].

Once inside the lymphoid tissue, naïve T-cells begin to sample peptide:MHC complexes on dendritic cells, looking for the right match. Once the naïve T-cell encounters a matching antigen on an activated antigen-presenting cell (APC), it loses its ability to exit the lymph node and stays in place to undergo clonal expansion and differentiation. This stalling in cell migration is mediated by the downregulation of the spingosine-1-phosphate receptor 1 (S1PR1). The spingosine-1-phosphate (S1P) concentration gradient between the lymph and the blood draws

resting naïve T-cells expressing S1PR1 back into the circulation. S1PR1 internalization is induced by upregulation of CD69, whose expression is induced by TCR signaling. Once T-cell activation levels go down, so does CD69 expression, leading to re-expression of S1PR1 and subsequent egress of the T-cell back into the bloodstream[311,312].

7.2 CD8 T-cell effector functions

Expanded differentiated antigen-specific CD8 T-cells, known as cytotoxic T-lymphocytes (CTLs), are fully equipped to seek out and kill infected host cells. CTLs are essential in controlling chronic viral infections and eliminating infected, defective or pre-cancerous cells. Two classes of weapons are present in CD8 T-cells' artillery: cytokines and cytotoxic proteins. Most well-known anti-viral cytokines secreted by CD8 T-cells are IFN-γ, tumor-necrosis factor-α (TNF-α), and IL-2 [313]. IFN-γ promotes immunoproteasome formation, upregulation of TNF-α receptors and Fas/Fas Ligand (FasL) expression, Type 1 CD4 helper T-cell differentiation, macrophage activation, and anti-viral gene expression in target tissues[314,315]. TNF-α induces vasodilation, facilitates leukocyte recruitment, and helps with optimal IFN-γ and IL-2 production in CD8 T-cells [316,317]. Furthermore, TNF-α can induce apoptosis in target cells via TNF-receptor 1 (TNFR1) [318]. Similarly to TNFR1, Fas and FasL are involved in regulation of apoptosis. Effector CD8 T-cell that express FasL can trigger the apoptotic pathway in target cells bearing the Fas receptor [319]. Known as death receptors, both TNFR1 and Fas initiate a caspase cascade upon ligand binding, which activate enzymes, cleave nuclear proteins, and cause chromosomal DNA fragmentation [320,321]. Apart from the extrinsic apoptotic pathway, initiated by binding of death ligands to death receptors, CTLs use cytotoxic effector molecules, perforin and granzymes, to kill virus-infected cells. Contained within specialized granules, where these enzymes are inactive due to acidic conditions, these proteins work cooperatively to get inside the target cell and force it to undergo apoptosis [322,323]. Perforin's function is to permeabilize the target cell's membrane. It does so by inserting itself into the phospholipid bilayer and polymerizing with other perforin monomers to form a channel, which facilitates granzyme entry into the cell [324]. Granzymes A and B are the most abundant of the human granzymes. Granzyme B can initiate a caspase cascade by activating caspase-3 and by cleaving its substrates[325,326]. Importantly, both Granzymes A and B can induce apoptosis in a caspase-independent manner[322,327,328].

7.3 Lung CD8 T-cells Show Poor HIV-specific Response:

As mentioned in the previous sections, chronic inflammatory environment and antigen stimulation leads to impaired CD8 T-cell function in lungs of PLWH, rendering them susceptible to opportunistic infections. Although some of the effector functions of CD8 T-cells in the peripheral blood do recover after ART initiation, this is not the case for the CD8 T-cells in the lungs[329,330]. Moreover, elevation in CD8/CD4 ratio contributes to non-AIDS-related morbidity[331]. These cells might also induce excessive expansion of other CD8 T-cells in the vicinity via T-cell receptor independent mechanisms, known as "bystander activation"[332,333]. Excessive expansion and immune activation consequently lead to accumulation of these functionally impaired CD8 T-cells displaying reduced proliferation, poor effector functions, and high expression of inhibitory receptors, such as PD-1, in the lung[332,334,335].

Impaired CD8 T-cell function may further contribute to lung reservoir persistence. Several studies have shown that these cells are required for HIV infection control. Once HIV-specific CD8 T-cells rise during acute infection, peak viremia begins to subside, meaning that these cells play a crucial part in viral control during primary infection[336,337]. Furthermore, SIV-infected Rhesus Macaques whose CD8 T-cells have been depleted, show increased plasma viremia, which is reversible with CD8 T-cell repopulation[338]. Moreover, HIV mutants that can escape the CD8 T-cell response appear early during infection and persist, further demonstrating that there is a strong evolutionary pressure posed on the virus yielding CD8 T-cell escape highly advantageous[339,340]. In a recent study, our team has demonstrated that pulmonary CD8 T-cells show lower perforin expression *ex vivo* compared with blood CD8 T-cells, regardless of HIV or smoking status[341]. Pulmonary CD8 T cells also showed significantly lower *in vitro* degranulation ability and less effective HIV-specific CD4 killing capacity than blood CD8 T cells, potentially contributing to a suboptimal anti-HIV immune response within the lungs.

7.4 Tissue-resident CD8 T-cells

During mucosal inflammation or infections, some CD8 T-cells migrate into non-lymphoid tissue where they persist as long-lived memory cells. Known as tissue-resident memory T-cells (Trm), these cells are essential for a rapid local immune response in case of re-infection and may even provide superior immune protection, compared to T-cells from blood circulation[342]. Trm cells' key characteristic feature is that they do not recirculate back into the blood unlike effector memory and central memory T-cell subsets[343]. Virtually present in all peripheral tissues, these cells' distinct migratory profile allows us to identify them by markers of tissue retention: CD69, CD103, and CD49a[344,345]. CD69 expression is concomitant with spimgosine-1-phosphate (S1P) receptor downregulation, which in turn mediates cell egress from the tissue into the efferent lymphatics along the S1P gradient[346,347]. CD103, also known as αE integrin, pairs with integrin β7 and act as a receptor for E-cadherin – a cell adhesion molecule that makes up adherence junction between epithelial cells[348,349]. In context of mucosal infections, CD103 allows CD8 to traffic and adhere to the epithelium, where they can remain as Trm and act as first line of defense in case of re-infection[350,351]. Lastly, CD49a, or α1 integrin, pairs with integrin β1 to form VLA-1 – a collagen receptor [348,352]. CD49a is not only crucial for cell binding to the epithelial basement membrane at mucosal surfaces, but is also needed for cell migration along collagen rich surfaces[353,354]. Furthermore, in concert with TNFRII, CD49a promotes CD8 T-cell survival in the airways[355]. Notably, CCR7 and CD62L, lymph node homing receptors described earlier, are not expressed on tissue-resident T-cells[356,357]. Other chemokine receptors that have been associated with Trm include CXCR6 and CX3CR1. The only known ligand for CXCR6 is CXCL6, which is highly expressed by pulmonary epithelium. Together they control airway CD8 T-cell pool replenishment by recruiting CD8 T-cells from the interstitium[358]. Similarly, CX3CR1, a fractalkine receptor, is involved in cell trafficking and is important for cell migration into the sites of inflammation. While CX3CR1hi CD8 T-cells have been characterized as effectors, CX3CR1int cells have been termed as a peripheral memory subset with a remarkable proliferative capacity that is chiefly responsible for surveying peripheral tissues[359]. Some mice studies have shown that a portion of $CX3CR1^{int}$ CD8 T-cells that also express Killer cell lectin-like receptor subfamily G member 1 (KLRG1), a co-inhibitory receptor for E-cadherin. In particular,

two different CD8 Trm populations have been defined: one originating from KLRG1- CX3CR1^{lo} and another from KLRG1+CX3CR1^{int} memory precursors[360,361]. Notably, the ExKLRG1 CD8 Trm show display higher cytotoxicity than Trm originating from KLRG1- cells. How these Trm subsets are distributed within the human lung and whether their ratio is affected by HIV and chronic inflammatory lung diseases remains unknown.

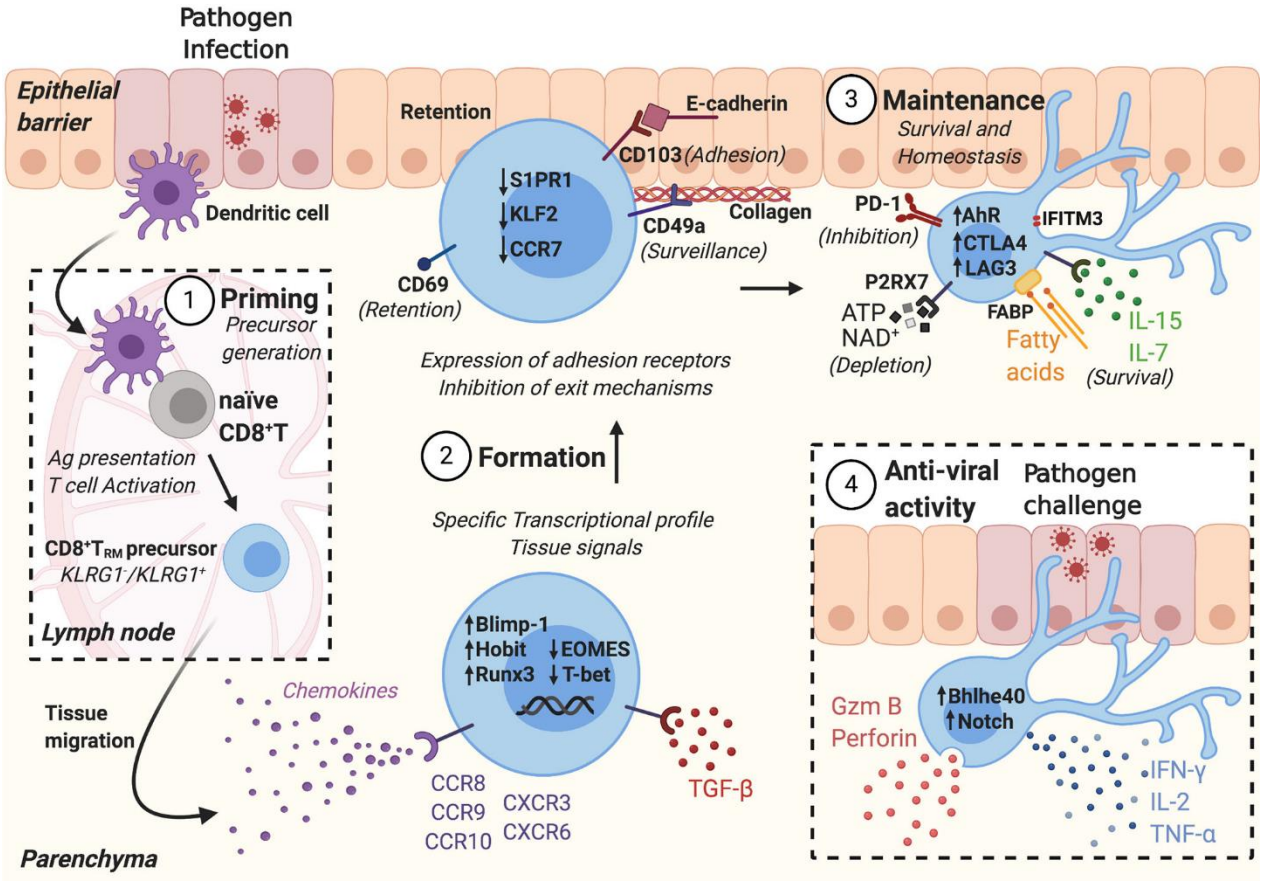

Figure 11: CD8 Trm formation and function are regulated at different stages. (1) Naïve CD8 T-cells are activated by a DC and form CD8 Trm precursors. (2) These precursor cells are recruited into peripheral tissues through chemokine signaling and a Trm transcriptional profile becomes activated through various tissue-specific signals. (3) CD8 Trm's primary role is to maintain homeostasis, which they do by sending and receiving inhibitory signals in absence of infection. (4) In the context of infection, CD8 Trm become activated and begin to exert their effector functions[362].

7.5 CD8 T-cell maintenance in small airways

Human pulmonary mucosal CD8 Trm dynamcis are still under investigation. The current model, developed in mice by Takamura and colleagues, proposes the following[363]. Once CD8 Trm within the pulmonary interstitium become reactivated, they upregulate their expression of CXCR6, which drives their subsequent migration to the airways along the CXCL16 gradient, a cytokine that is constitutively expressed by pulmonary epithelial cells. Some CD8 Trm undergo homeostatic proliferation in the pulmonary interstitium to replace cells that are lost during intraepithelial migration and biophysical removal. Takamura's group also suggest that lung CD8 Trm maintenance is largely independent of the CD8 Tem pool. They also note that interstitial CD8 Tem either leave the tissue via the draining lymph or migrate to the lumen in response to CXCL10 and other CXCR3 ligands[363]. Whether this model applies to the human lung, however, remains unknown. Thus, we wanted to verify some of these mechanisms in our human *ex vivo* data (Fig.12).

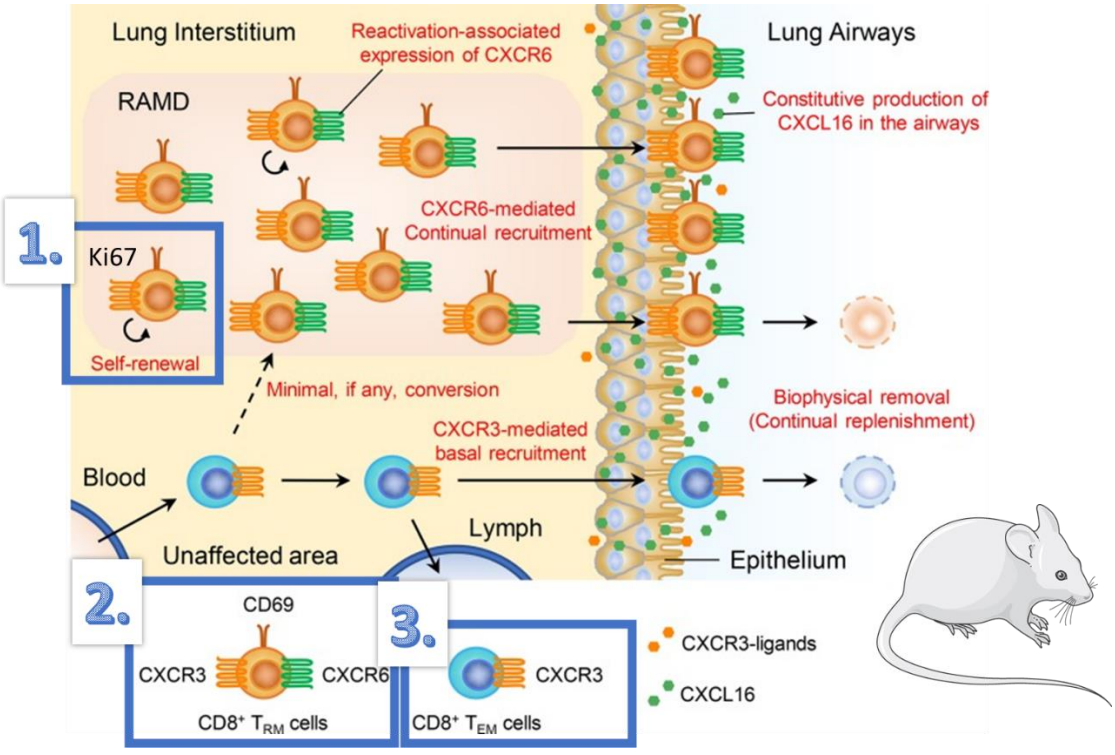

Figure 12: Lung CD8 T-cell maintenance model proposed by Takamura and colleagues. Mechanisms that we aimed to verify in our human data are highlighted in blue.

Based on currently available phenotypic data, we know that Trm cells, by definition, do not express KLRG1 and CX3CR1[358,359,361,364,365]. Buggert and colleagues have also shown that effector memory and terminally differentiated CD8 T-cells in the human efferent lymph are very different from cells found in the vasculature. They are less differentiated, very much like Tcm, lack cytotoxic effector proteins, and do not express CD69 or CX3CR1, while CX3CR1+ cytotoxic CD8 T-cells were confined to the vasculature, not only under physiological conditions but also during chronic HIV and Cytomegalovirus (CMV) infection[365]. Thus, we wanted to verify the phenotype and presence of blood-confined CD8 T-cells in our BAL CD8 T-cell pool (Fig.13).

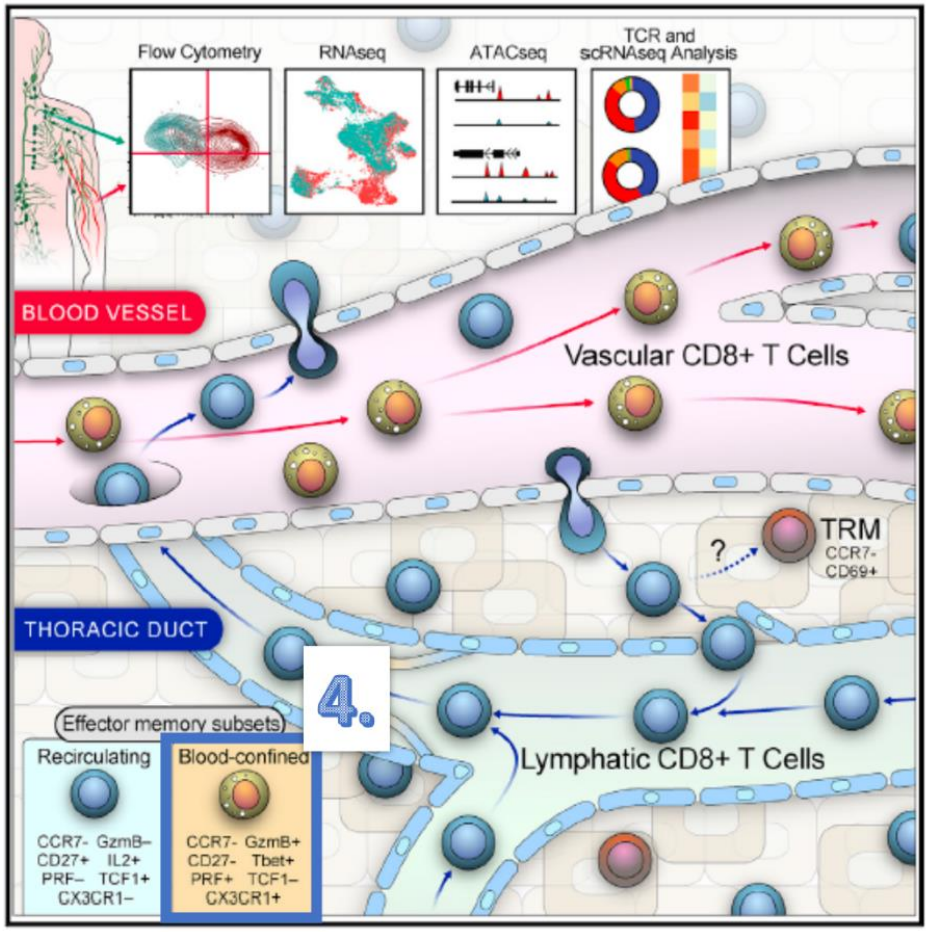

Figure 13: Characterization of recirculating T-cells from human thoracic duct lymph and their intravascular counterparts by Buggert *et al*[365]. We wanted to verify the phenotype and presence of blood-confined CD8 T-cells in our BAL CD8 T-cell pool (highlighted in blue).

7.6 Role of CD8 T-cells in Pulmonary Inflammation of PLWH

Evidently, the pulmonary environment is very different from the blood and other mucosal surfaces, such as the gastrointestinal tract. Unlike the blood, the pulmonary mucosa is exposed to high oxygen levels, airborne particles, and microbes. Furthermore, nutrients are quite scarce in this tissue, which in turn can affect CD8 T-cell metabolism and function[366,367]. Our lab has shown that CD8 T-cells in the small airways display low cytotoxicity regardless of HIV or smoking status, suggesting that lack of perforin within lung CD8 T-cells is part of a normal physiological cell state that potentially acts as a safeguard against undue tissue damage to a vital organ. Transcriptomic analysis performed in murine models has also shown that, in contrast to the cells from the pulmonary interstitium and the circulation, CD8 T-cells from the airways displayed lower expression of genes involved in cytotoxic function[366]. This CD8 Trm property during homeostatic conditions might be reversed in context of chronic inflammation, resulting in tissue destruction. Increased CD8 T-cell cytotoxicity has been documented in patients with chronic inflammatory lung conditions, such as smoking and COPD, both of which are common in Canada[267,368]. Furthermore, HIV is an independent risk factor for COPD development. In addition, lymphocytic alveolitis, characterized by infiltration of HIV-specific CD8 T-cell into the lung, is seen in up to 60% of untreated HIV infected persons in absence of lung tumors or respiratory infections[145,148,369]. Although ART has been very successful in transforming HIV from a death sentence into a manageable chronic condition, high prevalence of infectious and non-infectious pulmonary complications and co-morbidities in PLWH suggest that their lung immunity is not fully restored[177,282]. ART-treated PLWH are subject to persistent immune activation and chronic inflammation, which in turn leads to accelerated aging[370,371].

Our team has shown that HIV can persist within the lung mucosa despite long-term ART and that the lung HIV reservoir is larger compared to the HIV reservoir found in the blood[60]. We have also observed a significant reduction in proportions of senescent pulmonary CD28-CD57+ CD8 T-cells in HIV+ smokers, suggesting that differential CD8 T-cell dynamics are at play in these particular study groups[372]. Notably, smoking prevalence in PLWH is almost twice as high compared to the general population, which further adds to the low grade chronic systemic inflammation and decreased ART efficacy[373-375].

In summary, CD8 T-cell cytotoxic function is required for pathogen clearance and infection control but can wreak havoc if left unchecked. In the pulmonary mucosa, it remains unknown when and why CD8 T-cells lose their cytotoxic ability. Over the course of my MSc research project, we aimed to comprehensively characterize pulmonary CD8 Trm and non-Trm phenotypes and the epigenetic signature of their perforin promoter region in PLWH and seronegative controls and compare smokers to non-smokers within each of these study groups.

CHAPTER 8: Methods

8.1 Study population

The inclusion criteria for our study participants were the following: (1) have no ongoing respiratory disease or infection; (2) if HIV-infected, be on highly active ART with suppressed viral loads (≤40 copies/ml of plasma) and normalized CD4 T-cell counts (≥350 cells/mm3). Four groups (n=4-7/group) of participants were recruited at the McGill University Health Centre (MUHC): ART treated (suppressed VL ≥1 year and CD4 count ≥350 cells/mm3) smokers and non-smokers in addition to HIV-uninfected smokers and non-smokers. Many participants had already participated in previous studies at the clinic and had indicated their willingness to be re-contacted again in the future to hear about other recruiting studies by a member of the study team. Those who expressed interest in participating were called when the respirologist and study bronchoscopy dates became available. Participants were recruited based on meeting the inclusion criteria and their availability to undergo the procedure based on the dates available. It was not possible to match for age, gender, ethnicity, ART, or comorbidities due to the relatively small number of participants interested in volunteering for this type of study. Participants underwent spirometric testing several weeks prior to bronchoscopy to ensure the absence of any undiagnosed obstructive airflow disease. Participants were labelled as smokers if they smoked at least 1 tobacco cigarette daily. If the participant was a cannabis user but not a tobacco smoker, they were assigned to the non-smoker study group. 50-100 ml of BAL fluid were be obtained via bronchoscopies performed by a respirologist and matched 40ml blood samples were collected by venipuncture on the same day. Participants underwent BAL as a voluntary contribution to this study and not for any medical indication. They were compensated $160 for costs of travel and/or parking and the inconvenience of taking part in this study.

8.2 Ethics statement

This study has been ethically approved by the Research Institute of the McGill University Health Centre (#15-031), Université du Québec à Montréal (UQAM) (#602) and CHUM-Research Centre (#15-180) and procedures performed in accordance with the Declaration of Helsinki All participants provided written informed consent beforehand.

8.3 Lymphocyte isolation

Within 1 h following the bronchoscopy, BAL fluid was centrifuged and a portion of BAL cell pellets were collected for phenotyping and fluorescence-activated cell sorting. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using a lymphocytes separation medium and centrifugation, a technique which isolates lymphocytes through a density gradient. (Fig. 14)[376].

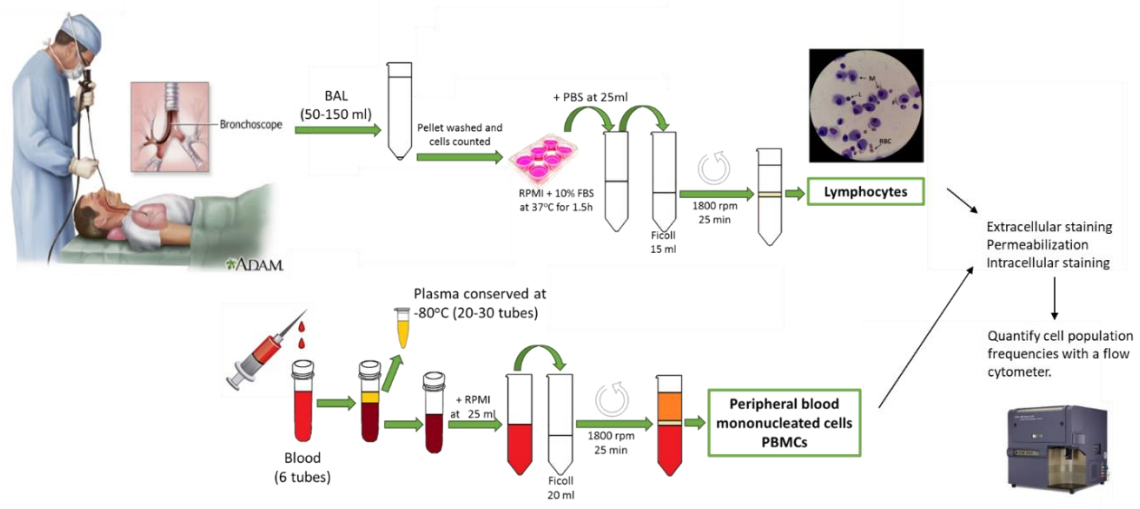

Figure 14: Isolation of pulmonary mucosal cells from bronchoalveolar lavage fluid (BAL) and peripheral blood mononuclear cells (PBMCs) from peripheral blood[376].

8.4 Flow cytometry

Live cells were used for phenotyping. All dead cells were excluded from the analysis using Live/Dead Aqua Stain Kit (ThermoFisher Scientific, Carlsbad, CA, USA). All extracellular staining was done in phosphate buffered saline 2% fetal bovine serum at 4°C for 1h. After extracellular staining was complete, the cells were washed and permeabilized using Transcription Factor

Staining Buffer Set (BD Bioscience, Mississauga, ON, Canada). Permeabilized cells were incubated with intracellular antibodies in a Perm/Wash buffer at 4°C for 1h and stored in PBS until acquisition. Pulmonary memory CD8 T-cells were characterized *ex vivo* using CD49a/CD69/CD103 (Trm markers)[377], CD45RA/CCR7/KLRG1 (T-cell subsets)[361,364], CX3CR1/CXCR6 (migration)[358,363], and granzyme-A/granzyme-B/perforin (cytotoxicity). Naïve CD8 T-cells (CD45RA+/CCR7+) were excluded from the analysis (Fig.15). The remaining memory cells were stratified based on expression of CD103 (E-cadherin receptor), CD69 (S1P1 signaling inhibitor), and CD49a (collagen IV receptor). CCR7- memory CD8 T-cells expressing at least one of these markers were defined as Trm. Memory CD8 Non-Trm were defined as CD103-CD69-CD49a-CCR7+/- cells. The antibodies used are listed in Table 1. A 5-laser BD Fortessa-X20 was used for acquisition and results were analyzed by FlowJo software V10.8.1 (BD Bioscience).

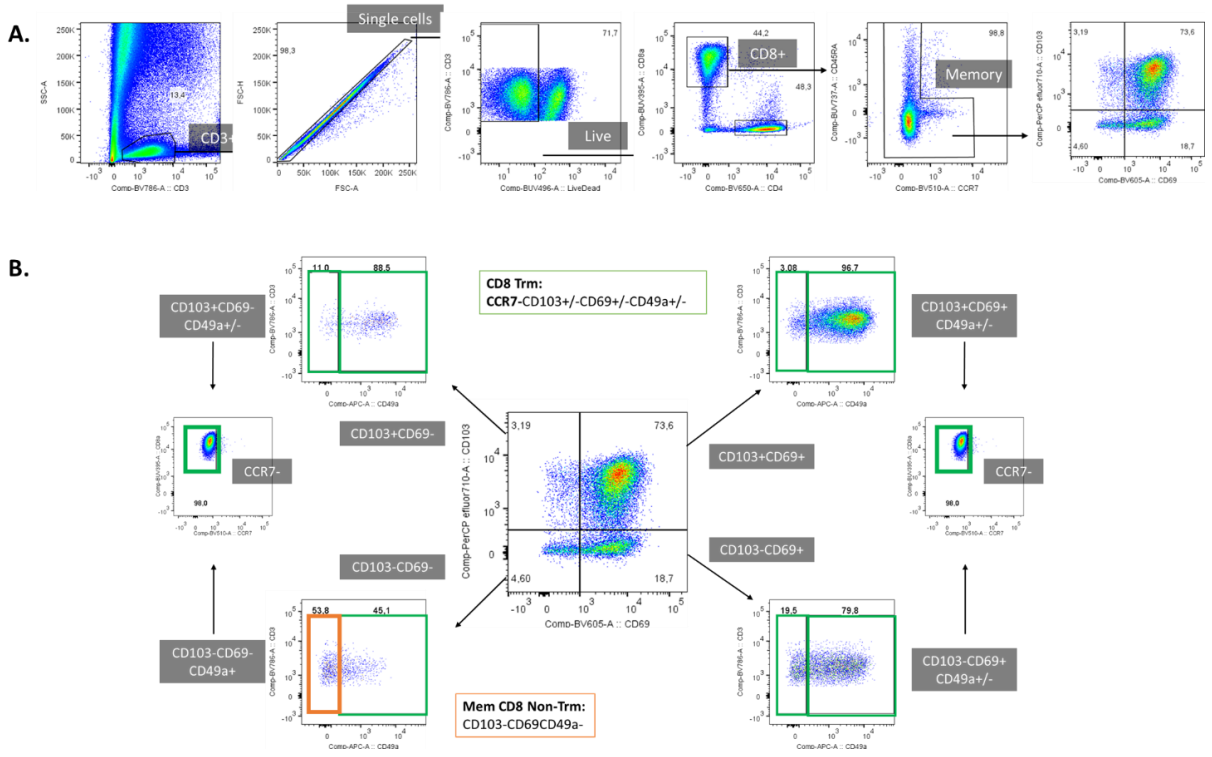

Figure 15: Gating strategy for tissue resident (Trm) and non-tissue resident (Non-Trm) CD8 T-cells from BAL fluid. **(A)** Live memory single CD8 T-cells were gated. **(B)** These were stratified into different CD8 T-cell subsets based on their expression of tissue-residency markers (CD69, CD103, CD49a). An additional $CCR7^{neg}$ gate was applied to all Trm subsets. Non-tissue resident CD8 Non-Trm were defined as $CCR7^{pos/neg}$ memory CD8 T-cells that do not express any of the tissue residency markers.

Table 1: Immunophenotyping markers used for characterization of BAL CD8 Trm.

Marker	Fluorochrome	Clone	Company	Description
LiveDead	350nm excitation	N/A	Invitrogen, Life Technologies Corporation, Eugene, OR	Dead cell marker
CD3	BV 786	SP34-2	BD Horizon, BD Biosciences, San Jose, CA	T-cell marker
CD4	BV 650	L200	BD Horizon, BD Biosciences, San Jose, CA	Used to gate on CD4 T-cells
CD8	BUV 395	RPA-T8	BD Horizon, BD Biosciences, San Jose, CA	Used to gate on CD8 T-cells
CD45RA	BUV 737	HI100	BD Horizon, BD Biosciences, San Jose, CA	Used to exclude naive T-cells
CD49a	APC	TS2/7	BioLegend, San Diego, CA	Collagen receptor
CD69	BV605	Fn50	BD Horizon, BD Biosciences, San Jose, CA	Prevents tissue egress
CD103	PerCP-eFluor710	B-Ly7	Invitrogen, Life Technologies Corporation, Eugene, OR	E-cadherin receptor
CXCR6 (CD186)	BV711	13B 1E5	BD OptiBuild, BD Biosciences, San Jose, CA	Lung homing marker (from interstitium to lumen)
CX3CR1	BV421	2A9-1	BD Horizon, BD Biosciences, San Jose, CA	Tissue homing marker (via enflamed endothelium)
CCR7 (CD197)	BV510	3D12	BD Horizon, BD Biosciences, San Jose, CA	Lymph node homing marker
CXCR3	R718	1C6/CXCR3	BD Horizon, BD Biosciences, San Jose, CA	Tissue homing
KLRG1	PE-CF594	14C2807	BioLegend, San Diego, CA	Co-inhibitory E-cadherin receptor
Granzyme A	PE	CB9	Invitrogen, Life Technologies Corporation, Eugene, OR	Serine proteases stored in granules of cytotoxic CD8 T-
Granzyme B	PE/Cy7	QA18A28	BioLegend, San Diego, CA	

				cells
Perforin	FITC	Pf-344	Mabtech, Stockholm, Sweden	Pore forming cytolytic protein
Ki67	APC eFluor780	SolA15	Invitrogen, Life Technologies Corporation, Eugene, OR	Marker of cell proliferation

8.5 Epigenetic analysis of *PRF1* promoter region

Memory and naïve CD8 T-cells were FACS sorted from peripheral blood mononuclear cells (PBMCs) based on their expression of CD3, CD8, CD28, and CD45RA for bisulfite sequencing on a 5-laser BD FACSAria Fusions (Table 2-3). Total CD8 T-cells were sorted from isolated BAL cells. CD8 DNA was isolated using the DNeasy kit (Qiagen, Hilden, Germany). Bisulfite conversion of DNA samples was performed using the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany).

Three CpG-rich regions upstream of the *PRF1* transcription start site, which included the upstream enhancer, the methylation sensitive region (MSR), and the proximal promoter, were amplified by PCR using primers targeting specifically bisulfite converted DNA[378] (Fig. 16). Primers were checked for any bias towards methylated/demethylated DNA. PCRs were carried out in 25 µl reaction volumes containing a final concentration of 1X PCR buffer (Platinum Taq DNA polymerase, Invitrogen), 1.5 mM MgCl2, 0.2 mM of dNTP mix, 0.4 µM forward and reverse primers, and 2-4 µl of bisulfite DNA template. The following thermocycling program was used: 95°C for 2 min, 40 cycles of 95°C for 30 s, melting temperature (Tm) for 40 s, 72°C for 1 min, and a final extension step of 72°C for 10 min. Tm values for each primer pair were the following: upstream enhancer – 58.1°C, MSR – 56.8°C, proximal promoter – 52.7°C. Following amplification, the quality of PCR products was assessed on a 2% agarose gel. The primers used are listed in Table 4.

PCR products were sent to a sequencing platform (Illumina MiSeq). Library preparation and sequencing were carried out at the Genomics Core of the CERMO-FC research center, UQAM. Bioinformatic analysis was done by the Bioinformatics Core Facility at the Institute for Research in Immunology and Cancer, Université de Montréal.

Table 2: Immunophenotyping markers used to sort BAL CD8 T-cells.

Marker	Fluorochrome	Clone	Company	Description
LiveDead	350nm excitation	N/A	Invitrogen, Life Technologies Corporation, Eugene, OR	Dead cell marker
CD3	BV 786	SP34-2	BD Horizon, BD Biosciences, San Jose, CA	T-cell marker
CD4	BV 650	L200	BD Horizon, BD Biosciences, San Jose, CA	Used to gate on CD4 T-cells
CD8	BUV 395	RPA-T8	BD Horizon, BD Biosciences, San Jose, CA	Used to gate on CD8 T-cells
CD45	PE/Cy7	HI30(RUO)	BD Pharmigen, BD Biosciences, San Jose, CA	Used to gate on haematopoietic cells

Table 3: Immunophenotyping markers used to sort naïve and memory CD8 T-cells from PBMCs.

Marker	Fluorochrome	Clone	Company	Description
LiveDead	350nm excitation	N/A	Invitrogen, Life Technologies Corporation, Eugene, OR	Dead cell marker
CD3	FITC	HIT3a	BD Pharmigen, BD Biosciences, San Jose, CA	T-cell marker
CD4	BV605	RPA-T4	BD Horizon, BD Biosciences, San Jose, CA	Used to gate on CD4 T-cells
CD8	PE-CF594	RPA-T8	BD Horizon, BD Biosciences, San Jose, CA	Used to gate on CD8 T-cells
CD45RA	APC-H7	5H9	BD Pharmigen, BD Biosciences, San Jose, CA	Used to distinguish between naïve and memory T-cells
CD28	APC	28.2	BD Pharmigen, BD Biosciences, San Jose, CA	

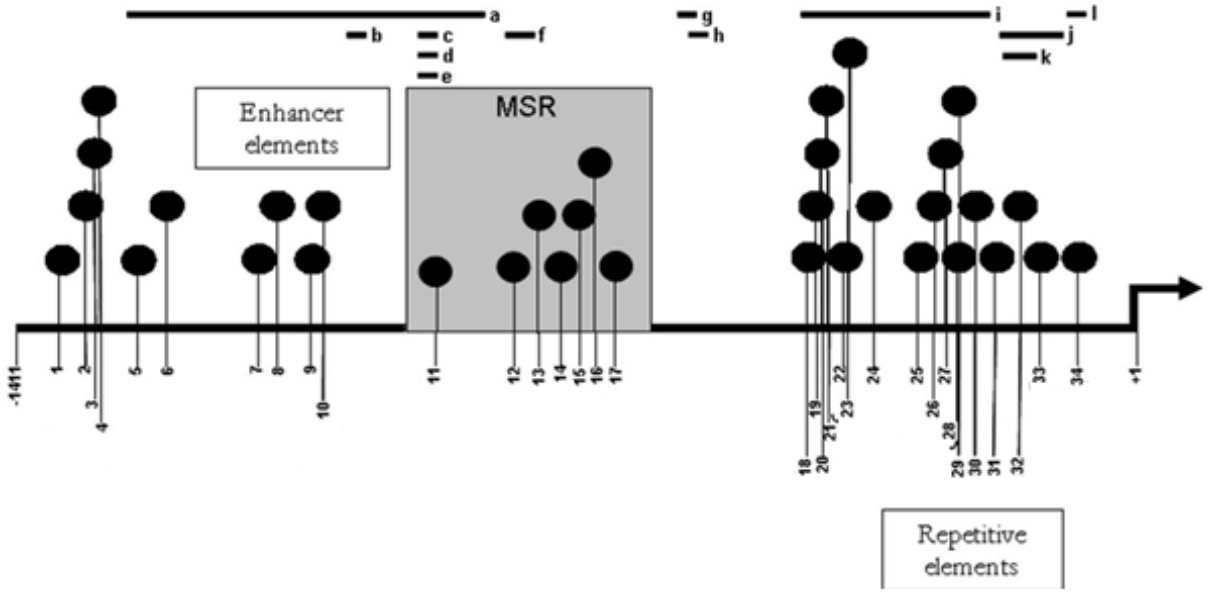

Figure 16: The perforin (*PRF1*) promoter region (1411 bp) with locations of 34 CpG. '+1' indicates the transcription start site. Ten CpGs are located in the upstream enhancer element region, seven in the methylation-sensitive region (MSR) and 17 in the proximal promoter (repetitive elements region). Transcription factor binding sites indicated: a) inducer response motif, b) γ-IFN responsive element, c) CRE element, d) AP-2 element, e) TPA-responsive element, f) STAT5-responsive enhancer, g) CCAT box, h) C-fos enhancer, i) 19 homologous repeats, j) three repeats, k) two repeats, l) GC box[378].

Table 4: *PRF1* promoter PCR primer sequences (5'-3') for bisulfite converted DNA.

CpG 1-10	**Forward**	GTTGGAAGGATGTGTAGAATTT
	Reverse	CTCCAAACCACATATAACATCA
CpG 11-18	**Forward**	GTGATTTATGAGATATGATGTTATATG
	Reverse	CTACTCAACCTACATCCCAC
CpG 19-34	**Forward**	TAGTTTATATTGTTGGTGTATAAT
	Reverse	CTCCTAAAATAATAACATCAACC

8.6 Statistical analyses:

GraphPad Prism V9 (San Diego, CA, USA) was used for statistical analyses. Wilcoxon matched-pairs signed rank test was used to compare paired and Mann-Whitney test was used to compare unpaired study variables.

CHAPTER 9: Results

9.1 Participant characteristics

Study participants were recruited through the Chronic Viral Illness Service (CVIS) at the McGill University Health Centre (MUHC). Most donors were Caucasian males (Table 1). There was no significant age difference between our study groups. All our HIV+ participants were on combination ART with suppressed viral loads (at least 1 year) and stable CD4 counts (median: 716 cells/mm^3). When grouped by smoking status, our smoker group had higher CD4 T-cell counts (median: 575 cells/mm^3 *vs* 996 cells/mm^3; $p<0.05$, Mann-Whitney test) but it was not significant after accounting for HIV status. Detailed characteristics for each participant can be found in Appendix A (Supplementary Table 1).

Table 5: Participant characteristics at the time of bronchoscopy

	N=19	
Demographic factors	**n=9 HIV+**	**n=10 HIV-**
Age, years (median, IQR)	54 (45.5, 60.5)	39 (28.0, 55.5)
Male sex, n (%)	8 (89)	9 (90)
Ethnicity, n (%) Caucasian Black/Haitian Asian Latino	 8 (89) 1 (11) 0 (0) 0 (0)	 8 (80) 0 (0) 1 (10) 1 (10)
HIV and immune-related factors		
Duration of HIV infection, years (median, IQR)	15 (2.5, 25.0)	N/A
Duration of time since viral load suppressed years (median, IQR)	4 (1.5, 6.0)	N/A
Antiretroviral regimen components, n (%) Integrase inhibitor Entry inhibitor NNRTI NRTI PI	 7 (78) 1 (11) 3 (33) 9 (100) 2 (22)	 0 (0) 0 (0) 3 (30) 0 (0)
CD4 count, cells/mm^3, median (IQR) CD4/CD8 ratio, median (IQR) CD8 count, cells/mm^3, median (IQR)	716 (486.0, 1137) 0.7 (0.6, 1.2) 836 (489, 1327)	822.5 (567.5, 1101) 1.8 (1.5, 2.2) 429.5 (342, 605)
Lifestyle factors		
Tobacco smoker, n (%) Yes No	 3 (33) 6 (67)	 3 (30) 7 (70)
Cannabis smoker, n (%) Current	 4 (44)	 4 (40)

9.2 Identifying major tissue-resident CD8 T-cell subsets

To have a broader view of lung mucosal CD8 Trm dynamics, the overall distribution of tissue-residency markers in BAL CD8 T-cells was evaluated. CD8 Trm cells were defined as CCR7^{neg} memory CD8 T-cells expressing at least one of the three Trm markers (CD69, CD103, CD49a). CD8 non-Trm were defined as all memory CD8 T-cells that are negative for all markers of tissue-residency evaluated in this study (Fig.15).

Across all study groups, nearly all BAL CD8 T-cells were positive for at least one Trm marker out of those included in our flow cytometry panel (Fig.16). Among these populations, the most prominent were CD69+ subsets (median % in total CD8 T-cells): CD103+CD69+CD49a+(median 55.00%), CD103-CD69+CD49a+ (median=14.00%), CD103+CD69+CD49a- (median=4.80%), CD103-CD69+CD49a- (median=7.29%) and the CD8 non-Trm subset CD103-CD69-CD49a- (median=4.69%). As the remaining CD8Trm subsets had very low frequencies (median ≤1% per subset) they were excluded from further analysis.

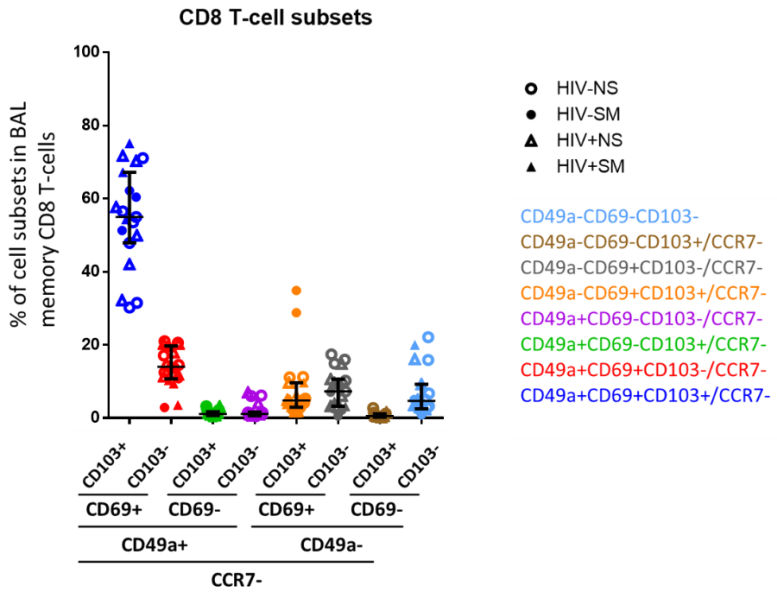

Figure 17: Frequencies of CD8 Trm subsets and CD8 Non-Trm in total BAL CD8 T-cells across all study groups. CD8 Trm subsets shown are CD103+CD69+CD49a+(median=55.00%), CD103-CD69+CD49a+ (median=14.00%), CD103+CD69+CD49a- (median=4.80%), CD103-CD69+CD49a- (median=7.29%), CD49a+CD69-CD103+ (median=1.07%), CD49a+CD69-CD103- (median=1.1%), CD49a-CD69-CD103+ (median=0.54%). CD8 non-Trm subset shown is CD103-CD69-CD49a-(median=4.69%).

9.3 Validating airway CD8 T-cell maintenance models in humans

9.3.1 CD8 Trm show high levels CXCR6/CXCR3 and lack CX3CR1/KLRG1:

Because most CD8 Trm dynamics models have been developed in murine models, we aimed to verify the most important mechanisms that have been put forth in the literature in our own human *ex vivo* phenotyping data prior to further analysis. Based on what has already been published, as we have described in Chapter 7 of the introduction, we came up with four hypotheses: (1) CD8 Trm show higher frequencies of CXCR6+CXCR3+ cells *vs* CD8 Non-Trm; (2) CD8 Non-Trm show higher frequencies of CXCR6-CXCR3+ cells *vs* CD8 Trm; (3) CD8 Trm *vs* Non-Trm show higher levels of proliferation; and (4) CD8 Non-Trm are CX3CR1+KLRG1+ Tem coming from the circulation[363,365]. The mechanisms corresponding to each hypothesis are highlighted in Fig.12-13.

Before stratifying different study groups, we evaluated the overall expression levels of CXCR6 and CXCR3 in different BAL CD8 T-cell subsets described earlier via flow cytometry and see if they match the lung CD8 T-cell migration model proposed by Takamura's group (Fig.12). After pooling our study groups together, we saw that CD103+ CD8Trm subsets showed the highest expression levels of CXCR6 and CXCR3 compared to other CD8 T-cell subsets. In fact, we saw that triple positive CD8 Trm (CD103+CD69+CD49a+) showed significantly higher expression levels of CXCR6/CXCR3, while cells that expressed two Trm markers or less, showed significantly lower CXCR6+CXCR3+ cell frequencies, with the lowest levels observed in CD8 Non-Trm cells (Fig.18A). The opposite pattern was observed in frequencies if CXCR6-CXCR3+ cells, where highest levels of these cells were present in CD8 Non-Trm compared to CD8Trm cell subsets (Fig.18B). This is in line with Takamura's airway CD8 Trm maintenance model, which postulates that CXCR6+CXCR3+ cells are part of the tissue-resident CD8 T-cell pool, while CXCR6-CXCR3+ cells are largely non-tissue resident.

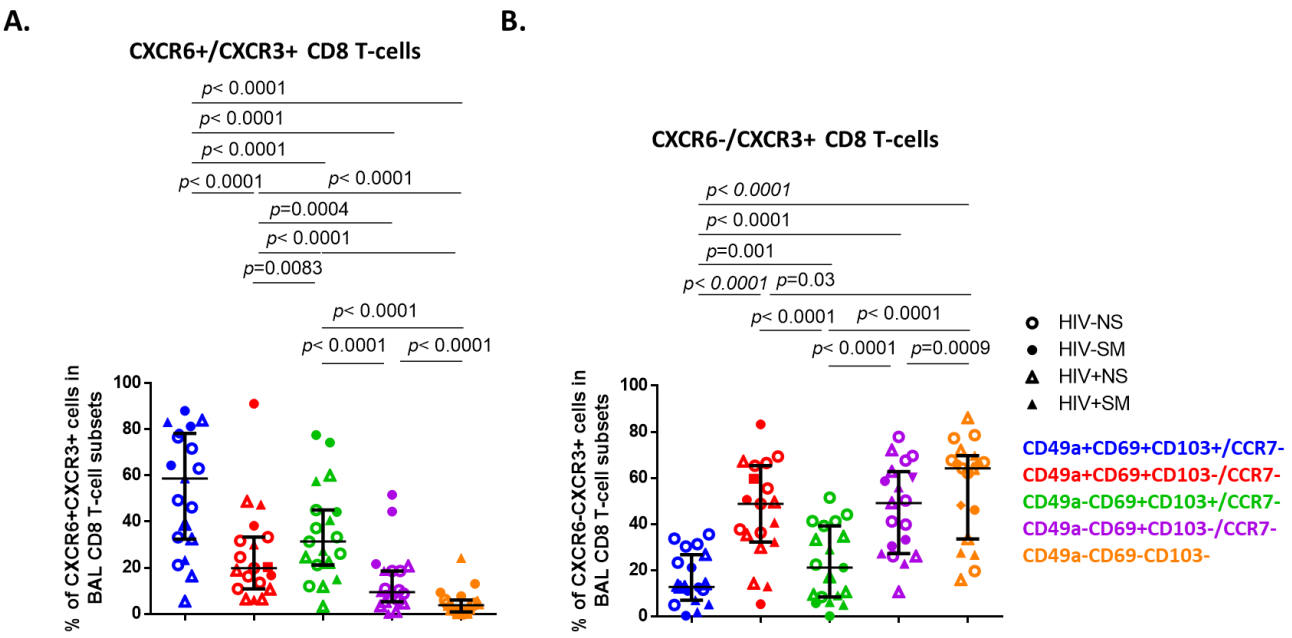

Figure 18: Frequencies of **(A)** CXCR6+CXCR3+ and **(B)** CXCR6-CXCR3+ cells among different BAL CD8 T-cell subsets assessed by flow cytometry (NS: non-smoker, SM: smoker). CD103+ CD8 Trm showed highest co-expression of CXCR6/CXCR3, while CD8 Non-Trm show significantly higher frequencies of CXCR6-CXCR3+ cells. (Wilcoxon matched-pairs signed rank test; HIV-NS: *n=7*; HIV-SM: *n=3*; HIV+NS: *n=6*; HIV+SM: *n=3*).

To assess the migratory origin of mucosal CD8 T-cells, we used CX3CR1 and KLRG1 as markers for inflationary CD8 T-cells (CD8 Non-Trm) homing into the alveolar space directly from the circulation (Fig.19)[358,359,361,364,365]. CD8 Non-Trm showed highest co-expression levels of these two markers, although the overall proportion of cells co-expressing these two markers was very low (median = 3.6%).

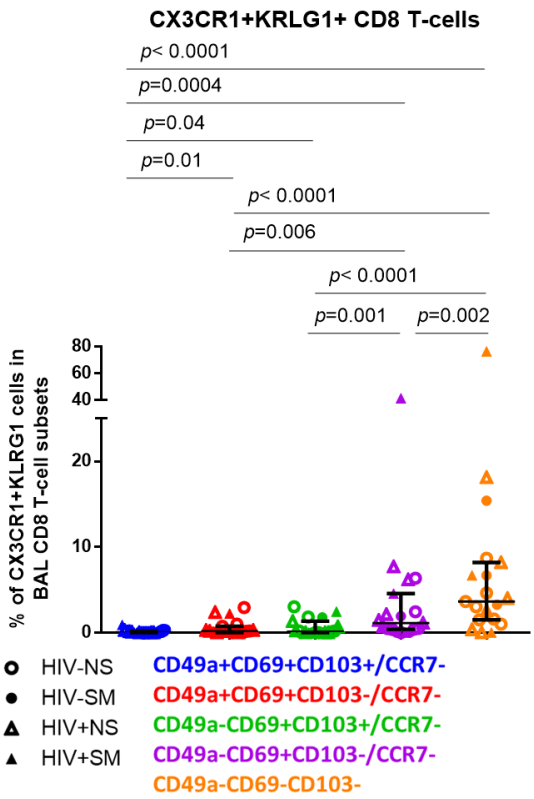

Figure 19: Co-expression of CX3CR1/KLRG1 among different BAL CD8 T-cell subsets assessed by flow cytometry (NS: non-smoker, SM: smoker). CD8 Non-Trm and CD69 single positive CD8 Trm show significantly higher frequencies of CX3CR1+KLRG1+ cells. (Wilcoxon matched-pairs signed rank test; HIV-NS: *n=7*; HIV-SM: *n=3*; HIV+NS: *n=6*; HIV+SM: *n=3*).

9.4.3 CD103+CD69+CD49a+ CD8 Trm show highest levels of Ki67

To evaluate proliferation levels of BAL CD8 T-cell subsets, as an indicator of their immune activation, we looked at their expression levels of Ki67 (Fig.20). Highest frequencies of Ki67+ cells were observed within triple positive CD8 Trm cells (CD103+CD69+CD49a+), indicating that these cells could have a marginally higher proliferative capacity compared to other CD8 Trm and Non-Trm subsets.

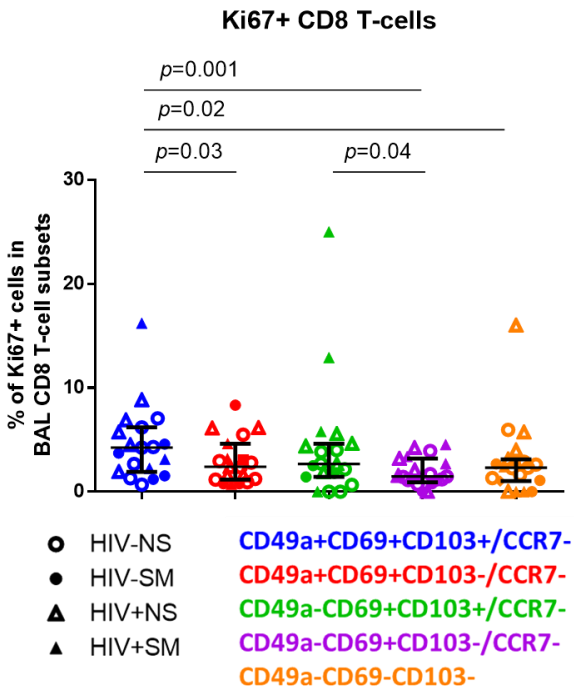

Figure 20: Expression levels of Ki67 among different BAL CD8 T-cell subsets assessed by flow cytometry (NS: non-smoker, SM: smoker). CD103+CD69+CD49a+ CD8Trm show significantly higher frequencies of proliferating cells compared to other CD8 t-cell subsets (Wilcoxon matched-pairs signed rank test; HIV-NS: *n=7*; HIV-SM: *n=3*; HIV+NS: *n=6*; HIV+SM: *n=3*).

9.5 Evaluating the effects of HIV and smoking on lung CD8 T-cell cytotoxicity and dynamics

9.5.1 Smoking promotes increased CD8 Trm migration and retention in the pulmonary mucosa:

Both smoking and positive HIV status were found to be independently associated with significantly higher CD8 T-cell frequencies and a lower CD4/CD8 T-cell ratio in BAL (Fig.21A-B) (Data pooled with patients described in this study and our previous paper[341]. Methods and characteristics for the added participants can be found in Appendix A: Supplementary Tables 2-3 and Supplementary Methods). Furthermore, smoking, but not HIV status, was associated with higher frequencies of CD103+ memory CD8 T-cells (Fig.21C-D). Although we did not see a significant difference in frequencies of CD103+CD8Trm subsets between our study groups when they were stratified by both HIV and smoking status (Fig.22A,C), we did observe a significant reduction in proportions of CD103-CD8Trm subsets between our smoking and non-smoking participants (Fig.22B,D). The same observation was made when grouping the data by smoking status but not by HIV status (Fig.23). Smoking status was also associated with higher

frequencies of CXCR6+CXCR3+ CD8 T-cells within our HIV- study groups, suggesting increased transepithelial migration of interstitial CD8Trm into the lumen (Fig.24). This difference was not as pronounced between HIV+ smokers and non-smokers, which means that HIV and smoking have differential effects on lung CD8 T-cell dynamics.

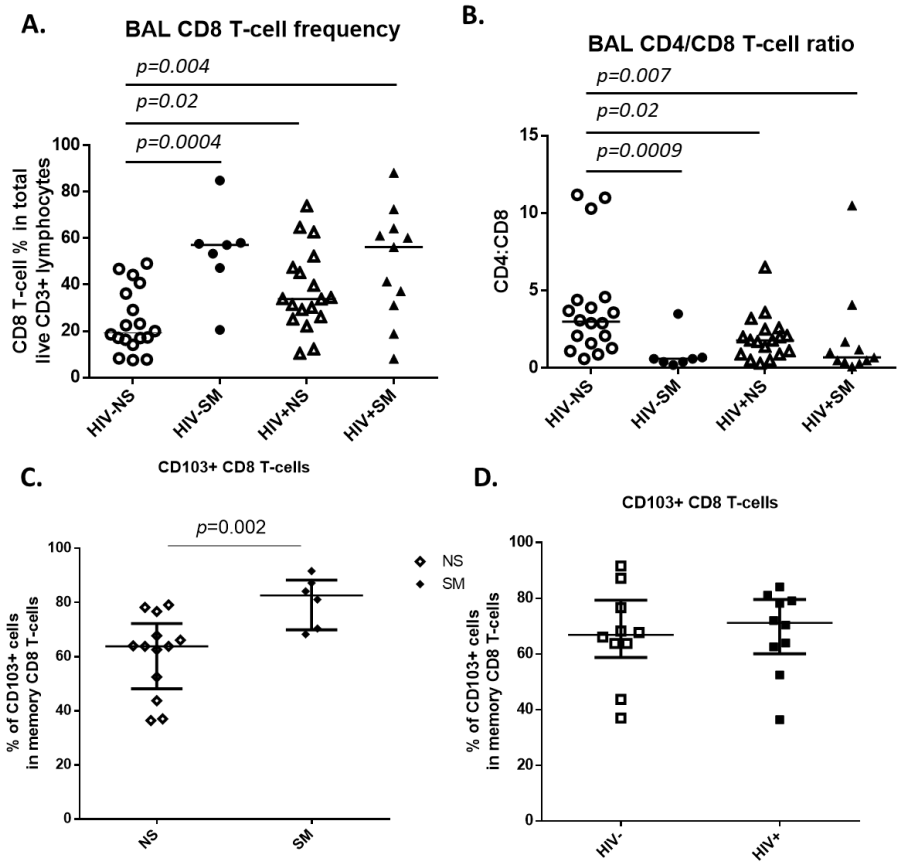

Figure 21: Smoking promotes CD8 T-cell retention in pulmonary mucosa (NS: non-smoker, SM: smoker). **(A)** CD8 T-cell frequencies in live CD3+ lymphocytes from bronchoalveolar lavage fluid. Both smoking and positive HIV status were independently associated with significantly higher CD8 T-cell frequencies and **(B)** a lower CD4/CD8 T-cell ratio in BAL (Mann–Whitney rank-sum test; HIV-NS: *n=18*; HIV-SM: *n=7*; HIV+NS: *n=18* HIV+SM: *n=11*). Smoking **(C)**, but not HIV status **(D)**, were associated with a higher frequency of CD103+ memory CD8 T-cells (Mann–Whitney rank-sum test; HIV-NS: *n=7*; HIV-SM: *n=3*; HIV+NS: *n=6*; HIV+SM: *n=3*).

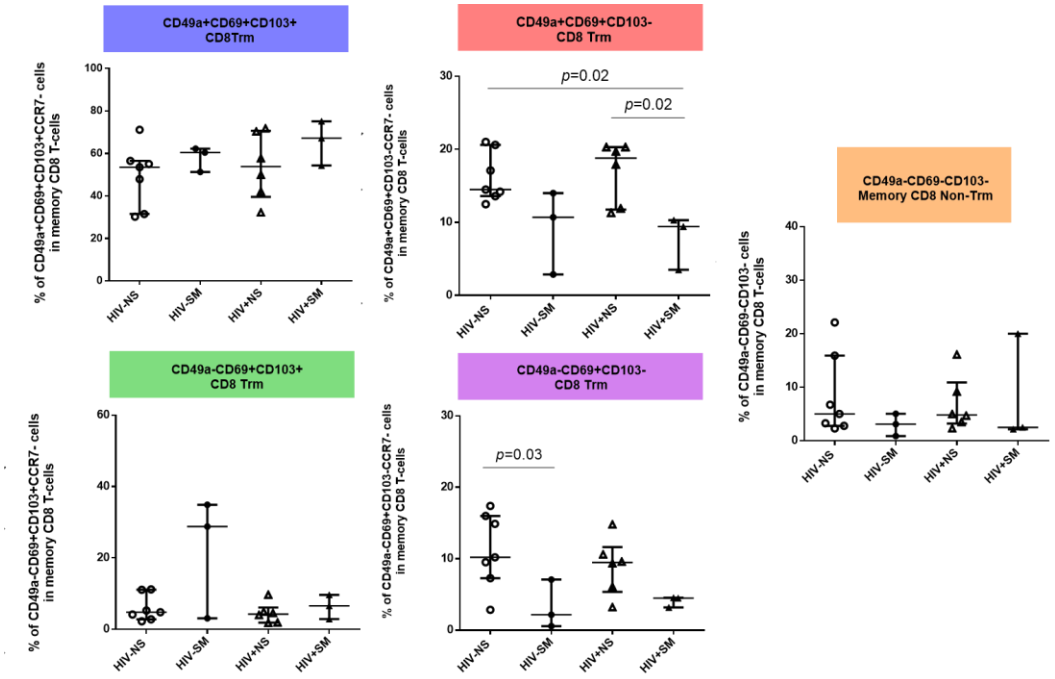

Figure 22: Frequencies of BAL CD8Trm and Non-Trm subsets across different study groups. (NS: non-smoker, SM: smoker; Mann–Whitney rank-sum test; HIV-NS: *n=7*; HIV-SM: *n=3*; HIV+NS: *n=6*; HIV+SM: *n=3*).

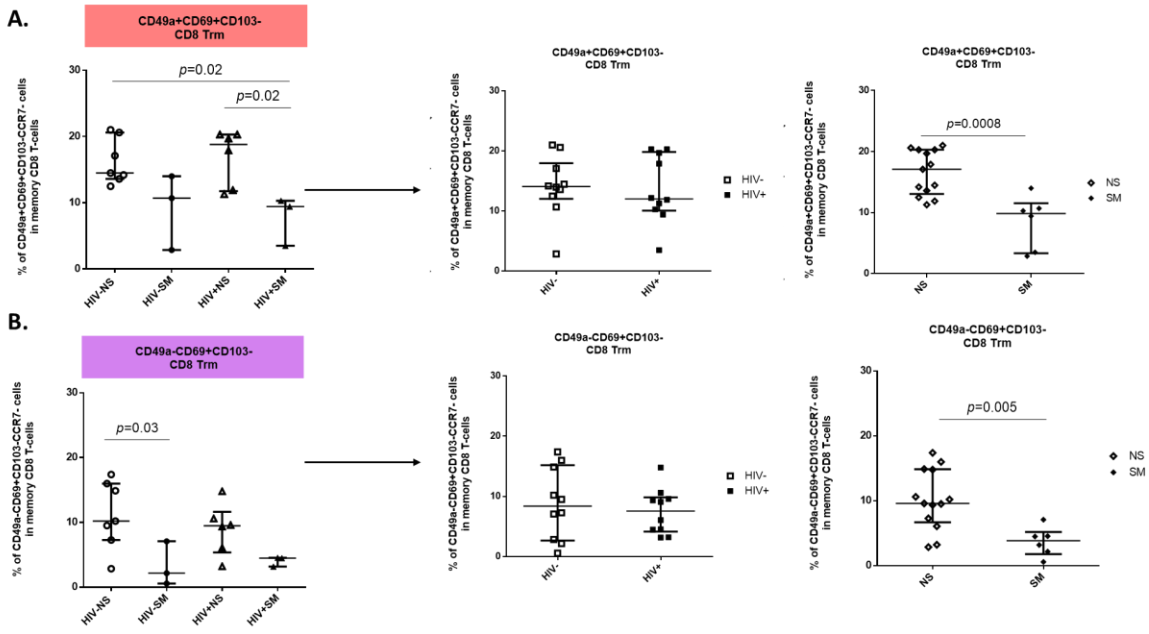

Figure 23: Frequencies of **(A)** CD103-CD69+CD49a+ and **(B)** CD103-CD69+CD49a- CD8Trm subsets in BAL stratified by either smoking or HIV status alone. (NS: non-smoker, SM: smoker; Mann–Whitney rank-sum test; HIV-NS: *n=7*; HIV-SM: *n=3*; HIV+NS: *n=6*; HIV+SM: *n=3*).

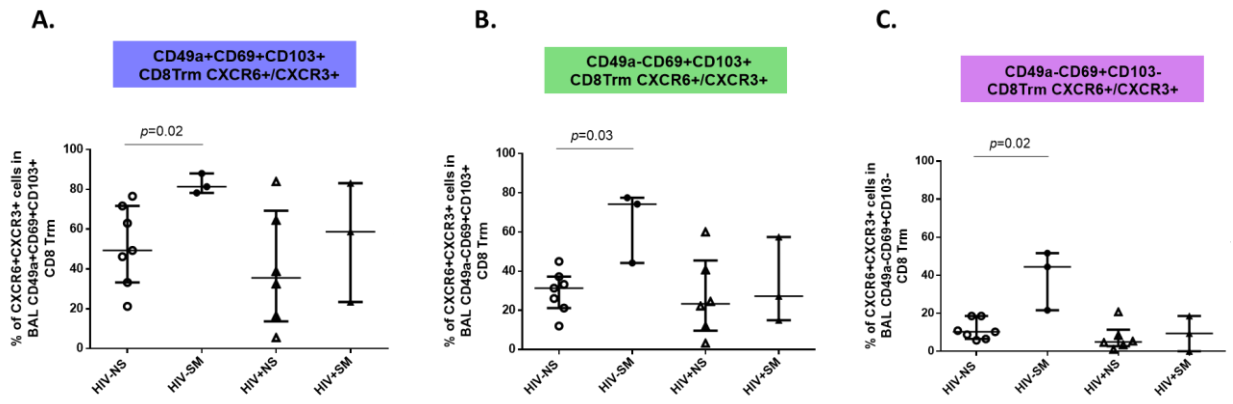

Figure 24: Frequencies of CXCR6+CXCR3+ cells within different CD8Trm subsets. **(A)** CD103+CD69+CD49a+, **(B)** CD103+CD69+CD49a-, and **(C)** CD103-CD69+CD49a- CD8Trm subsets are shown. No significant differences in CD103-CD69+CD49a+ CD8Trm were observed (data not shown). (NS: non-smoker, SM: smoker; Mann–Whitney rank-sum test; HIV-NS: *n=7*; HIV-SM: *n=3*; HIV+NS: *n=6*; HIV+SM: *n=3*).

9.5.2 HIV infection is associated with increased infiltration of cytotoxic CD8 T-cells in BAL:

To evaluate differences in cytolytic potential of lung CD8 Trm and non-Trm subsets, we examined their expression of granzymes A and B, as well as perforin. Interestingly, memory CD8 Non-Trm of HIV+ donors showed significant differences compared to seronegative controls. We observed higher levels of granzymes A/B, perforin, and KLRG1 in HIV+ *vs* HIV- non-smokers (Fig.25). Furthermore, some of the CD8 Trm subsets also showed significantly higher GzmB expression, which was once again associated with HIV+ status (Fig.26). This suggests that HIV infection could promote increased migration of cytotoxic CD8 T-cells into the airways and retention of their cytotoxic abilities upon tissue-residency establishment. Additionally, one of the CD69 single positive CD8Trm subsets (CD103-CD69+CD49a-) comprised of significantly higher frequencies of CX3CR1+KLRG1+ cells (Fig.27), highlighting that these CD8 T-cells might have recently migrated from the peripheral circulation. Lastly, CD49a- CD8 Trm subsets displayed higher levels of Ki67 in HIV+ non-smokers *vs* HIV- non-smokers, meaning that increased CD8 T-cell frequencies observed in BAL of PLWH could be caused by both increased

migration from the vasculature and augmented proliferation of specific CD8Trm subsets (Fig.28).

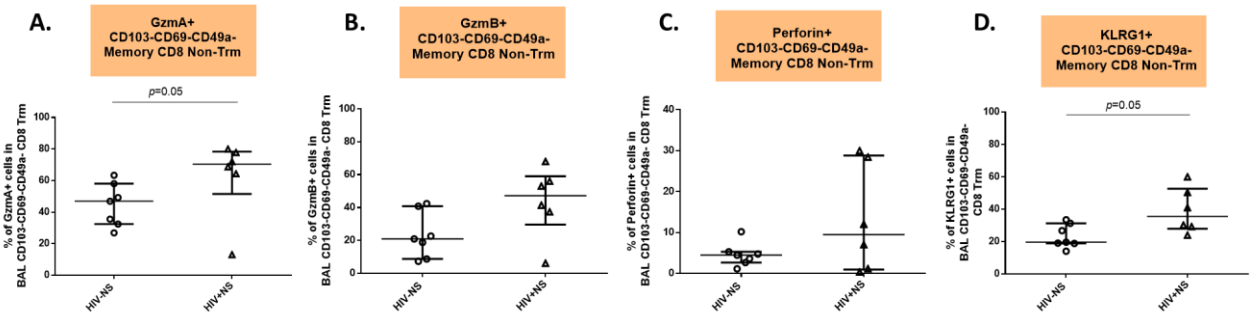

Figure 25: Expression of **(A)** GzmA, **(B)** GzmB, **(C)** Perforin, and **(D)** KLRG1 in BAL memory CD8 Non-Trm. (NS: non-smoker, Mann–Whitney rank-sum test; HIV-NS: *n=7*; HIV+NS: *n=6*).

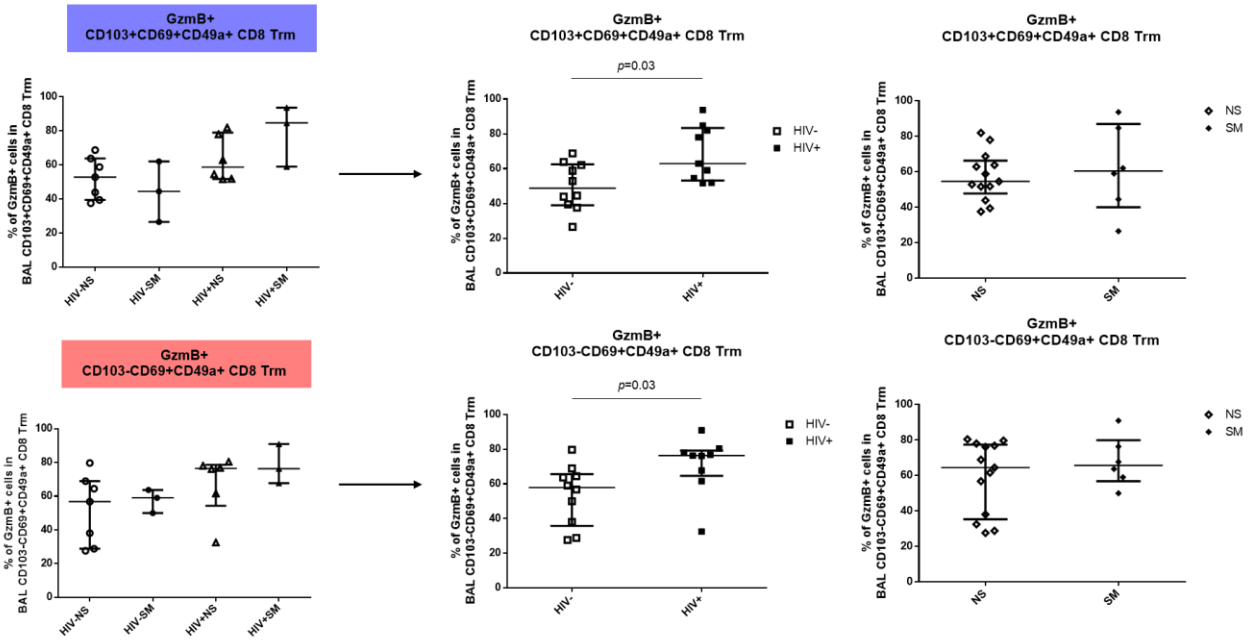

Figure 26: Frequencies of GzmB+ cells within CD69+CD49a+ CD8Trm subsets. (NS: non-smoker, SM: smoker; Mann–Whitney rank-sum test; HIV-NS: *n=7*; HIV-SM: *n=3*; HIV+NS: *n=6*; HIV+SM: *n=3*).

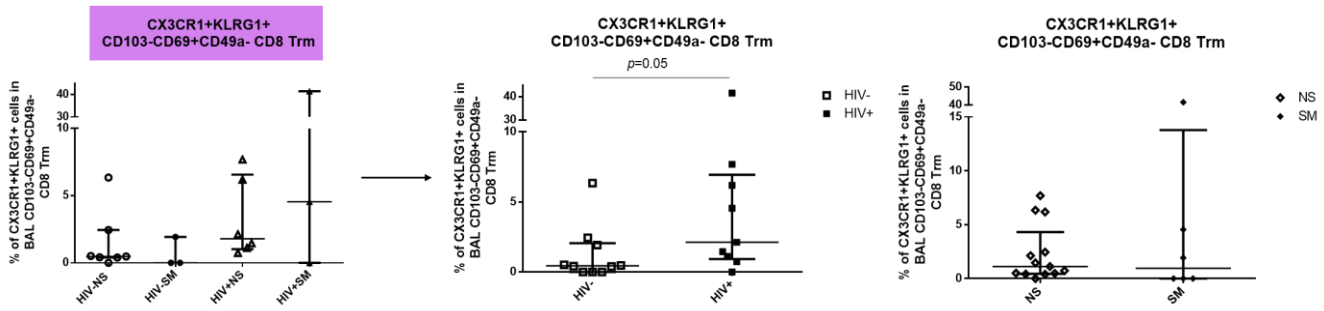

Figure 27: Frequencies of CX3CR1+KLRG1+ cells within CD103-CD69+CD49a- CD8Trm. Data points were regrouped by either HIV or smoking status alone. (NS: non-smoker, SM: smoker; Mann–Whitney rank-sum test; HIV-NS: *n=7*; HIV-SM: *n=3*; HIV+NS: *n=6*; HIV+SM: *n=3*).

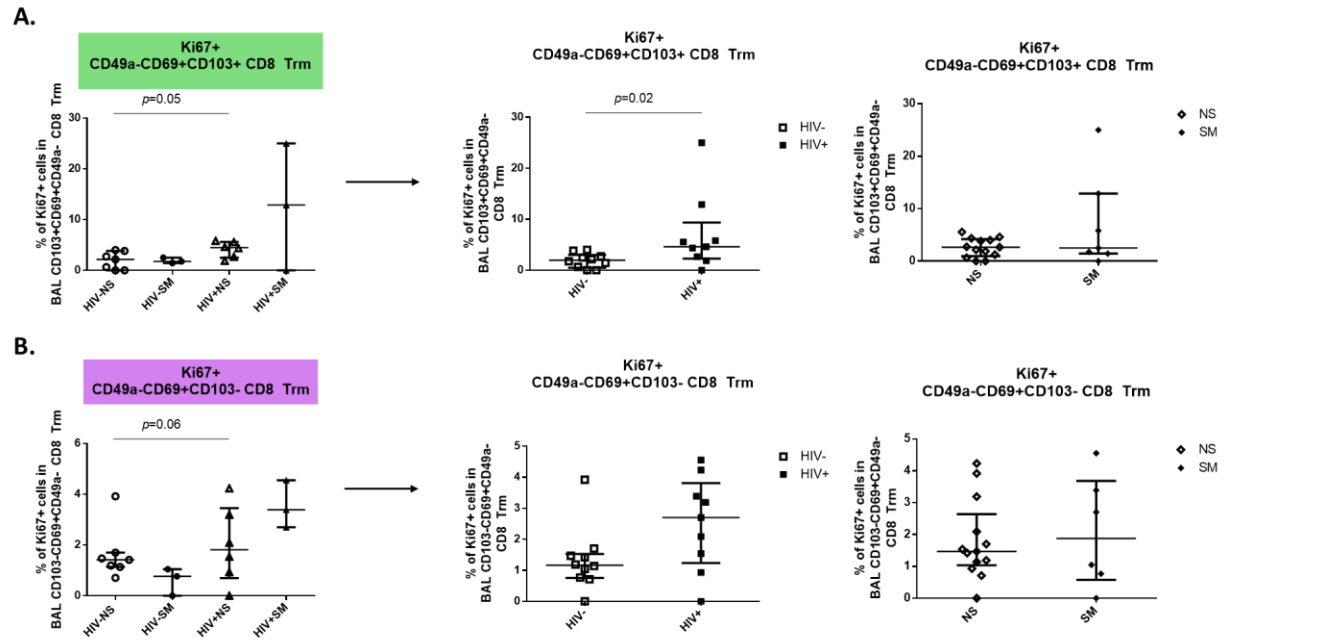

Figure 28: Frequencies of Ki67+ cells within different CD49a-CD8Trm subsets. **(A)** CD103+CD69+CD49a- and **(B)** CD103-CD69+CD49a- CD8Trm subset is shown. No significant differences in CD103-CD69-CD49a- CD8 Non-Trm was observed (data not shown). (NS: non-smoker, SM: smoker; Mann–Whitney rank-sum test; HIV-NS: *n=7*; HIV-SM: *n=3*; HIV+NS: *n=6*; HIV+SM: *n=3*).

9.6 Assessing the epigenetic signature of the *PRF1* promoter region

In our previous study performed on total BAL CD8 T-cells, we observed uniformly low perforin expression in lung mucosal CD8 T-cells compared to CD8 T-cells from the blood[341]. To determine whether low cytotoxicity of these cells is programmed at the epigenetic level, we performed bisulfite sequencing of the perforin promoter on total CD8 T-cells sorted from BAL, as well as both naïve and memory CD8 T-cell populations sorted from PBMCs.

We hypothesized that lung CD8 Trm may lack perforin expression due to epigenetic silencing of the *PRF1* promoter region. Unexpectedly, *PRF1* promoter of BAL CD8 T-cells was largely demethylated compared to naïve and memory CD8 T cells from the blood, suggesting that perforin suppression likely isn't caused by methylation of the promoter but rather takes place either at transcriptional or translational levels (Fig.29). In other words, lung CD8 T-cell cytotoxicity likely isn't determined during their differentiation and commitment to the Trm cell lineage. Instead, it is likely dictated by the airway environment, which changes their transcriptomic and metabolic processes that shut down perforin expression.

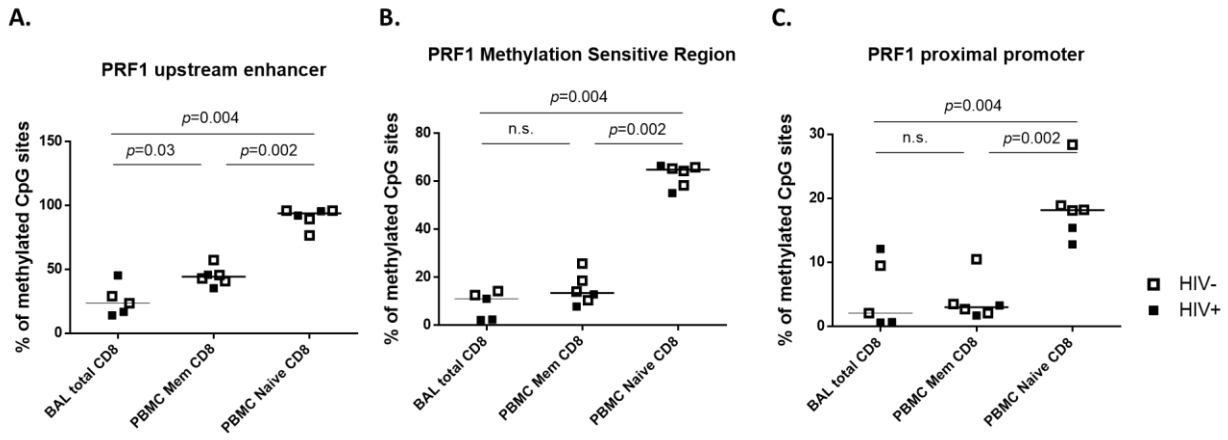

Figure 29: Frequencies of methylated CpG sites within three different regions of the *PRF1* promoter: (A) upstream enhancer, (B) the methylation sensitive region, and (C) the proximal promoter. (Mann–Whitney rank-sum test; BAL: HIV- *n=2*, HIV+ *n=3*; PBMC: HIV- *n=4*, HIV+ *n=2*.)

CHAPTER 10: Discussion

In this study, we provide one of the first reports on the phenotypic characteristics of multiple CD8 Trm subsets in context of HIV and smoking. Our data is consistent with the notion that CD8 Trm maintenance in the small airways is largely independent of circulating CD8 Tem and are replenished by CXCR6+CXCR3+ CD8 Trm homing to the airway lumen from the interstitium[358,363]. Furthermore, we have shown that both smoking and HIV dysregulate pulmonary mucosal CD8 T-cell dynamics but in distinct ways. Firstly, we have evaluated the expression of cell surface markers related to four CD8 T-cell migration mechanisms using multiparametric flow cytometry (Fig.12-13)[363]. The following hypotheses based on the current lung CD8 T-cell migration model were tested: 1) CD8 Trm show higher frequencies of CXCR6+CXCR3+ cells *vs* CD8 Non-Trm; (2) CD8 Non-Trm show higher frequencies of CXCR6-CXCR3+ cells *vs* CD8 Trm; (3) CD8 Trm *vs* Non-Trm show higher levels of proliferation; and (4) CD8 Non-Trm are CX3CR1+KLRG1+ Tem coming from the circulation. These hypotheses are supported by our *ex vivo* human lung data. CD8 Trm showed higher frequencies of CXCR6+CXCR3+ cells compared to non-Trm cells, meaning that human lung mucosal CD8 T-cells are largely derived from the interstitial CD8 Trm cell pool. As mentioned in chapter 7 of the introduction, CXCR6 has been described in mice as a receptor that helps partition CD8 Trm cells within different lung compartments, recruiting them from the interstitial space to the airway lumen[358,363]. Furthermore, both murine and human data show that CXCR6 is largely absent in circulating CD8 T-cells[358].

In line with Takamura's model, our data show that CD8 non-Trm display higher frequencies of CXCR6-CXCR3+ cells compared to CD8 Trm cells. However, their frequencies were also significantly higher in CD103-CD8Trm *vs* CD103+CD8Trm. In other words, the CXCR6-CXCR3+ phenotype does not solely apply to CD8 non-trm and likely cannot be used as a sole marker for inflationary CD8 T-cells that immigrate directly from the vasculature. It is possible that CD103+CD8Trm which express CXCR6 could be derived from CD103-CD8Trm, which upregulate the expression of both CD103 and CXCR6 in response to environmental stimuli. For instance, TGF-β is a well-known pleiotropic cytokine that induces CD103 expression on CD8 T-cells and

promotes Trm formation[379]. Importantly, without TGF-β signaling, inflationary CD8 T cells are unable to upregulate CD103 and fail to differentiate into CD103+ CD8 TRM cells in the skin and gut of mice and don't remain in the tissue for long periods of time[351,380,381].

There have been different reports regarding Ki67 expression by CD8Trm *vs* CD8 from the peripheral blood. While some groups report that lung and other human Trm cells exhibit lower expression levels of Ki67 compared with T cells in blood, others document the exact opposite[364,382]. In our own data, the median of Ki67+ cells was lower in circulating memory CD8 T-cells compared to BAL CD8 Trm subsets, although those differences were not significant (data not shown). Nonetheless, most agree that the human Trm cell pool is at least partially maintained via low-level homeostatic *in situ* proliferation in the absence of infection rather than solely being replaced by circulating T-cells[363,377,383,384]. In our pooled data set, we observed significantly higher levels of Ki67 in triple positive CD8 Trm, although the difference in Ki67 expression seems to be marginal and thus it is hard to tell whether a 1-2% increase in Ki67+ cells is biologically significant. Given the environmental differences between the pulmonary interstitium and the alveolar space, further experiments are required to compare proliferative capacities of various CD8Trm subsets that reside in different lung compartments[366].

Because CX3CR1 and KLRG1 have repeatedly been shown to be expressed by circulating but not tissue-resident T-cells, we propose that CX3CR1 and KLRG1 could be used as markers of inflationary CD8 T-cells in tissues[359,361,364,365]. Our data show that BAL CD8 Non-Trm have the highest frequencies of CX3CR1+KLRG1+ cells, which are almost entirely absent within CD8 Trm subsets expressing CD103 or CD49a. However, the overall proportion of cells co-expressing these two markers was very low (median = 3.6%) suggesting that either expression of CX3CR1/KLRG1 does not encompass all CD8 Non-Trm from peripheral circulation or that expression of either CD103, CD69, or CD49a does not encompass all CD8 Trm cells in human lower airways. In other words, it is hard to predict the origin of CD103-CD69-CD49a- cells that do not express KLRG1 or CX3CR1 with *ex vivo* cell phenotyping data alone.

Of note, whether CD69 single positive CD8 T-cells (CD69+CD103-CD49a-) could be considered as a 'pure' tissue-resident cell subset is also under question. Some studies claim that CD69

functional requirement in generation and maintenance of CD8 Trm can vary significantly depending on the tissue. Walsh and his group, for example, have demonstrated in multiple mouse models that CD69 does not play a significant role in generation of Trm in the small intestine, while being critical for establishment of tissue-residency in the kidney[385]. Furthermore, its expression is not unique to CD8 Trm and it is frequently used as a marker of activation, since CD69 expression is rapidly induced on the cell surface upon T-cell receptor engagement[386].

After stratifying different study groups, we have observed that HIV and smoking status have distinct and independent effects on lung mucosal CD8 T-cell dynamics. We have found that, while both HIV and smoking are independent factors that can promote higher CD8 T-cell frequencies in BAL, they do so through different mechanisms. Our results suggest that smoking could promote increased CD8 Trm migration via CXCR6 and augmented retention in the pulmonary mucosa through CD103, while HIV could promote infiltration of activated CD8 T-cells (CX3CR1+KLRG1+CD103-CD69+CD49a-) from the periphery.

We have also found that HIV infection is linked with higher granzyme A/B expression in lung CD8 T-cells. Increased granzyme expression observe in our HIV+ study group is likely linked to residual immune activation that is well-known in PLWH. Furthermore, we have previously shown that lung CD8 T-cells of PLWH are highly activated (HLA-DR+) and exhausted (PD-1+) compared to their blood counterparts[341]. It is well-known that chronic inflammation during HIV infection is one of the biggest contributors to immune dysfunction, co-morbidities, and replenishment of the viral reservoir even under ART. Furthermore, as mentioned previously, our team has also demonstrated that HIV can persist within the lung mucosa despite long-term ART and that lung HIV reservoir is larger compared to the blood[341]. Thus, it is possible that increased CD8 T-cell infiltration and granzyme expression is caused by residual virus hiding in pulmonary immune cells, such as CD4 T-cells and AMs. Several mechanisms have been put forth to explain HIV reservoir persistence after many years of ART treatment: poor ART penetration into deep tissues, residual replication, persistent cell stimulation due to residual antigen load, and CD8 T-cell exhaustion[63,64,179]. Residual replication is of particular importance as it can create a self-perpetuating cycle where some of the virus will continue replicating, increasing viral

antigen load locally[65,66]. In turn, this antigen load will stimulate nearby immune cells, activating their transcriptional machinery, which will produce more virus if that cell harbors intact and inducible viral DNA.

The size of the viral reservoir is associated with residual levels of immune activation in ART-treated PLWH. Levels of both cell-associated RNA and proviral DNA have been positively correlated with frequencies of activated (CD38+HLA-DR+) and exhausted (PD-1+) CD4+ and CD8+ T cells [67,68]. Although most of these studies are typically done in the blood, the lungs of PLWH show a similar phenomenon. All lung T-cells show high frequencies of activation (HLA-DR) [60,129,341]. Lung CD4 T-cells from treated PLWH also show higher frequencies of senescent cells (CD57), while CD8+ and double negative T-cells show high expression of PD-1[60,129,341]. Furthermore, Collini and colleagues were able to detect residual levels of HIV viral protein gp120 in BAL fluid of HIV-1-seropositive donors with median ART treatment time of 75 months[187]. This finding is of particular importance, because the ability to detect HIV viral proteins in PLWH who have been on ART for over 6 years suggests that there is ongoing residual viral replication in that tissue that contributes to chronic pulmonary inflammation and subsequent CD8 T-cell activation. These granzyme-producing CD8 T-cells will not be able to remove HIV-infected cells without perforin, which is largely lacking in BAL CD8 T-cells regardless of HIV or smoking status, as we have demonstrated in one of our recent studies[341,387-390]. However, granzymes can exert other functions in extracellular space, such as extracellular matrix degradation[391]. Importantly, increased granzyme expression has been associated with increased COPD severity, which could help explain why PLWH are at a higher risk of COPD and emphysema development even if they do not smoke[392,393].

In contrast, we did not see any differences in granzyme or perforin levels between our smoking and non-smoking participants, unlike in already published literature documenting increased CD8 T-cell cytotoxicity in the blood and airways of smokers[392,394,395]. Several study limitations might have impeded us in this regard, which includes a small sample size and potentially large variation in the contents and quantities of what our participants smoke, as well as how often they smoke. Notably, all our tobacco-smoking participants were also either current or occasional cannabis smokers. This could be one of the reasons we didn't see a significant

difference in cytotoxic effector molecule expression between our smoking and non-smoking study groups since tetrahydrocannabinol, the main psychoactive compound in marijuana, has been previously shown to suppress CD8 T-cell cytolytic activity *in vitro* an *in vivo*[396,397]. Our finding that smoking leads to increased retention of CD8 T-cells in the lung, however, is supported by Corleis *et al* in their recent report, where they document increased CD8 T-cell levels in endobronchial brushings of HIV-1 infected smokers[398]. We further show that this is likely caused by increased recruitment of lung interstitial CD8 Trm to the alveolar space via CXCR6, which has also been implicated in COPD and lymphocytic alveolitis[399,400]. Notably, Freeman and others report that increased CXCR6 expression by CD8 T-cells is positively correlated with COPD severity, meaning that CXCL16/CXCR6 axis blockade might constitute a new therapeutic approach for mitigating some of the damage seen in smoking COPD patients[401].

Lastly, to determine whether low perforin expression by lung CD8 T-cells is programmed at the epigenetic level, we assessed the epigenetic state of the perforin promoter on total CD8 T-cells sorted from BAL, as well as both naïve and memory CD8 T-cell populations sorted from PBMCs.

We hypothesized that lung CD8 Trm may lack perforin expression due to epigenetic silencing of the *PRF1* promoter region, which is the case in the blood. In fact, we saw a significant positive correlation between levels of *PRF1* methylation and perforin expression in naïve and memory CD8 T-cells isolated from PBMCs (data not shown). In contrast, this correlation was entirely absent in BAL CD8 T-cells and we report that *PRF1* promoter region is highly demethylated in this tissue. Therefore, it is unlikely that perforin expression is suppressed at the epigenetic level in BAL CD8 T-cells. This supports our hypothesis where it is the pulmonary environment and metabolic shift that lead to perforin suppression. Compared to the blood, pulmonary mucosa is exposed to high oxygen levels, airborne particles, and microbes, and where nutrients are scarce, which can in turn affect CD8 T-cell metabolism and function[366,367]. It is also in-line with some previous transcriptomic studies, which report that naïve CD8 T-cells undergo multiple DNA demethylation events upon cognate antigen recognition, some of which are permanent. Youngblood and colleagues have shown that the *PRF1* locus of LCMV-specific memory CD8 T-cells in their mouse model remained demethylated even though these memory cells did not

express high levels of perforin. Similarly, Akondy *et al* have published similar evidence on human Yellow Fever Virus – specific CD8 T-cells isolated from vaccinated human participants. This open chromatin profile at effector gene sites, which is maintained in memory CD8 T cells isolated several years after vaccination, indicates that they retain an epigenetic fingerprint of their effector history and remain poised for a quick response upon pathogen re-exposure. However, the nature of this 'quick response' must be controlled by tissue environment. In our previous study, we have performed CD3/CD28 stimulation on both total BAL cells and PBMCs and although we did see an increase in perforin levels in BAL, it wasn't nearly as pronounced as in CD8 T-cells taken from the blood, suggesting that other perforin-suppressing mechanisms that regulate lung CD8 T-cell cytotoxicity must be further downstream[366,402,403].

Evidently, this study has several limitations. Firstly, our data only include cell phenotypes and epigenetic analysis of the perforin promoter region. No functional assays or transcriptomic analyses have been performed. Thus, further experiments are required to support the activity we have tentatively ascribed to CD8 Trm subsets in smokers and PLWH. We would like to perform single cell RNA sequencing in the future to have a much more comprehensive picture of each CD8 T-cell subsets and their differences based on smoking and HIV status of our study participants. Secondly, our cells were obtained exclusively from the airways, which means that (1) we do not know how their phenotype differs from cells residing in the pulmonary interstitium and (2) our cells come from different environments within the airways, which include alveoli, bronchioles, and bronchi. As briefly mentioned in Chapter 2 of this thesis manuscript, these are different mucosal compartments, which differ in cell and surface fluid composition and thus might represent different immunological niches[404,405]. However, in this regard, interparticipant variation was minimized as bronchoscopy was performed by the same respirologist for all our participants using a standardized procedure[376].

CHAPTER 11: Future Directions

ART does not fully restore lung immunity in PLWH, who continue to suffer from high burdens of infectious and non-infectious pulmonary illnesses[197]. Many different factors contribute to pulmonary immune perturbations in PLWH even during ART. Inflammation is the biggest driver of pulmonary pathologies and lung HIV reservoir persistence in these individuals[60,68,406]. High blood flow, large pools of target cells, close cell-to-cell proximity, and small arteriole size likely contribute to lung HIV infection and spread[177]. Furthermore, because the lung mucosa is continuously exposed to many airborne antigens, these could further stimulate residual HIV replication and proliferation of infected cells[187].

Smoking is highly prevalent among PLWH, but HIV and smoking exerts differential effects on lung CD8 T-cell dynamics[407]. To further detangle their effects, single cell transcriptomic analysis stratifying different CD8 Trm subsets is required. Importantly, no cell exists in a vacuum, and other lung cell, should also be examined in all our study groups. Evaluating CD4+ Treg function would be of particular importance given that these immunoregulatory cells have been implicated in suppression of CD8 T-cell cytotoxic function, pulmonary fibrosis, and CD8 Trm establishment[408-410]. To confirm that increased CD8 T-cell migration in smokers is indeed caused by CXCR6 and CXCR3, levels of their ligands in BAL supernatant should be measured. To better understand the effects of smoking on lung CD8 T-cell cytotoxicity, incubation of total BAL cells from HIV- non-smokers with various concentrations of cigarette smoke extracts should be performed. Similarly, cannabis smoking is also very common amongst PLWH, with over 50% of PLWH endorsing current cannabis use in the United States and Canada[411,412]. Therefore, analogous studies could be conducted using various concentrations of cannabis extracts or phytocannabinoids. Lastly, evaluating the effects of HIV and smoking on lung microbiome and its subsequent effect on CD8 Trm subset distribution and function would be of great interest.

The overall lung CD8 T-cell maintenance model remains under investigation. We have yet to fully understand the difference in environmental effects between the alveolar space and the pulmonary interstitium in the human lung. A more reliable CD8 Trm marker for human studies

is also required, since some of Trm cells do not express any of the tissue-residency markers that are currently used in the literature[413,414].

Given the importance of mucosal CD8 T-cells in immune defense and inflammation, a comprehensive understanding of the dynamics, regulation, and functions of pulmonary mucosal CD8 T-cells in PLWH and uninfected individuals, as well as the impact of smoking, will contribute towards the design of therapeutic strategies for pulmonary inflammation. Furthermore, this research is particularity timely given that the outcomes might have broad implications in our understanding of immunological susceptibility to lung infections, including coronaviruses, and non-infectious pulmonary diseases in addition to mucosal vaccine strategies, which aim to generate a robust CD8 T-cell response.

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APPENDIX A

Supplementary Table 1: Detailed participant characteristics. All clinic patients undergo Tuberculin Skin Test (TST) testing at initial clinic visit as part of routine care. Abbreviations: PMHx, Past medical history; PCP, Pneumocystis carinii pneumonia; VL, viral load.

ID	Age, years	Sex, M/F	Ethnicity	PMHx of PCP or Tuberculosis (active or latent)*	Duration HIV (years)	Comorbidities	Tobacco	Cannabis	CD4 count/ mm^3	CD4/CD8 ratio	CD8 count/mL	Nadir CD4 count/mL	ART regimen at time of bronchoscopy	Undetectable VL (years)
People living with HIV														
AMV03	52	M	Caucasian	No	21	1) Hypertenston 2) Dyslipidemia 3) Gastroesophageal reflux disorder 4) Depression	No	No	382	0.7	477	300	Isentress/ Truvada	4
AMV13	54	F	Caucasian	No	22	1)Lipodystrophy 2)Previous Hepatitis C (cured)	Yes	Yes	716	0.6	1285	unavailable	Isentress/ Reyataz/ Complera	5
AMV15	51	M	Caucasian	No	15	1)Coronary artery disease	No	No	468	0.4	836	216	Triumeq	2
AMV17	41	M	Caucasian	No	4	none	Yes	Yes	1267	0.9	1369	595	Viread/Edurant/ Tivicay	3
AMV23	33	M	Caucasian	No	1	none	Yes	Yes	633	1.5	472	633	Genvoya	1
AMV26	50	M	Caucasian	No	18	1)Depression	No	No	471	0.5	870	356	Atripla	4
AMV27	59	M	Caucasian	No	1	None	No	No	501	0.6	626	341	Isentress/ Descovy	1
Patient35	62	M	Caucasian	No	28	1)Hypertension	No	Yes	899	1.8	501	Unavailable	Triumeq	9
Patient45	57	M	Black-Haitian	No	8	1)Diabetes Type 2 2)Diabetic nephropathy 3)Hypertension 4)Iron-deficiency anemia 5) cataracts	No	No	1007	0.7	1543	295	Ritonavir, Darunavir, Maraviroc, Abacavir	7
Seronegative controls														
AMC 09	55	M	Caucasian	No	N/A	None	Yes	Yes	1173	3.3	355	N/A	N/A	N/A
AMC11	27	M	Caucasian	No	N/A	None	No	No	439	1.3	302	N/A	N/A	N/A
AMC 18	28	M	Caucasian	No	N/A	None	No	No	826	1.8	621	N/A	N/A	N/A
AMC 19	53	M	Caucasian	No	N/A	1)Hypertension 2)Impaired glucose tolerance	No	No	692	3.0	232	N/A	N/A	N/A
AMC20	34	M	Asian	No	N/A	None	No	No	545	404	1.3	N/A	N/A	N/A
AMC 21	57	M	Caucasian	No	N/A	1)Depression	No	No	1077	1.9	455	N/A	N/A	N/A
AMC 22	42	F	Caucasian	No	N/A	None	Yes	Occasionally	1248	1.9	678	N/A	N/A	N/A
AMC 23	65	M	Caucasian	No	N/A	None	Yes	Occasionally	819	1.8	456	N/A	Truvada 21 jours/mois	
AMC 25	28	M	Caucasian	No	N/A	None	No	No	575	1.5	383	N/A	Truvada daily	N/A
AMC 27	36	M	Latino	No	N/A	None	No	Occasionally	835	1.6	600	N/A	Truvada daily	N/A

Supplementary Table 2: Characteristics of additional participants included in Figure 21 A,B. Abbreviations: IQR, interquartile range; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor. The *p* values were obtained by Fisher exact tests for categorical variables and Mann–Whitney rank-sum test for continuous variables.

	N=43		
Demographic factors	**n=26 HIV+**	**n=17 HIV-**	***p*-value**
Age, years (median, IQR)	53 (50.0, 58.0)	55 (29.0, 60.5)	0.78
Male sex, n (%)	22 (84.6)	16 (94.1)	0.63
Ethnicity, n (%) Caucasian Black/Caribbean Black/African	 22 (84.6) 2 (7.7) 2 (7.7)	 17 (100) 0 (0) 0 (0)	0.40
HIV and immune-related factors			
Duration of HIV infection, years (median, IQR)	17.5 (12.0, 24.3)	-	n/a
Duration of time since viral load suppressed, years (median, IQR)	8.3 (3.0, 11.3)	-	n/a
Antiretroviral regimen components, n (%)* Integrase inhibitor NNRTI PI	 20 (76.9) 6 (23.1) 6 (23.1)	-	n/a
CD4 count, cells/mm^3, median (IQR) CD4/CD8 ratio, median (IQR) CD8 count, cells/mm^3, median (IQR)	363.5 (390.8, 754.3) 0.6 (0.5, 1.1) 621.8 (520.5, 1142.3)	634.5 (433.5, 947.0) 2.0 (1.5, 3.4) 332.0 (160.5, 492.5)	0.57 <0.001 <0.001
Pulmonary co-infection/opportunistic infection history Previous Pneumocystis pneumonia Previous Mycobacterium avium pneumonia Kaposi's sarcoma Latent Tuberculosis infection	 2 (7.7) 1 (3.8) 1 (3.8) 1 (3.8)	-	n/a
Lifestyle factors			
Tobacco smoker, n (%) Yes No	 11 (42.3) 15 (57.7)	 7 (41.1) 10 (58.8)	1.00
Cannabis smoker, n (%) Current	 6 (23)	 1 (5.9)	0.21

Supplementary Methods

Study population, sample collection, and cell isolation

ART-treated PLWH smokers (n = 11) and nonsmokers (n = 15) and uninfected smokers (n = 7) and nonsmokers (n = 10) were recruited without any respiratory symptoms or active infection at the McGill University Health Centre (Montreal, QC, Canada) (Supplementary Table 2). Participants underwent spirometric testing several weeks prior to bronchoscopy to ensure the absence of any undiagnosed obstructive airflow disease. For each participant, data were captured on age, sex, ethnicity, cannabis use, and tobacco smoking at the time of bronchoscopy. Participants were labeled as smokers if they smoked at least one tobacco cigarette daily. Additionally, for participants living with HIV, data were captured on duration of HIV infection, time since viral load suppression, components of their antiretroviral regimens, and history of pulmonary infections. HIV-infected participants were all ART treated with suppressed plasma viral load and CD4 count higher than 350 cells/mm3 for at least 3 y. A total of 50 to 100 ml of BAL fluid was obtained via bronchoscopy, and 40 ml of blood was collected from each participant. BAL cells and PBMCs were isolated.

Ethical considerations

The study was approved by the Institutional Review Boards of the McGill University Health Centre (no. 15-031) and Université du Québec à Montréal (no. 602). All study participants signed a written informed consent.

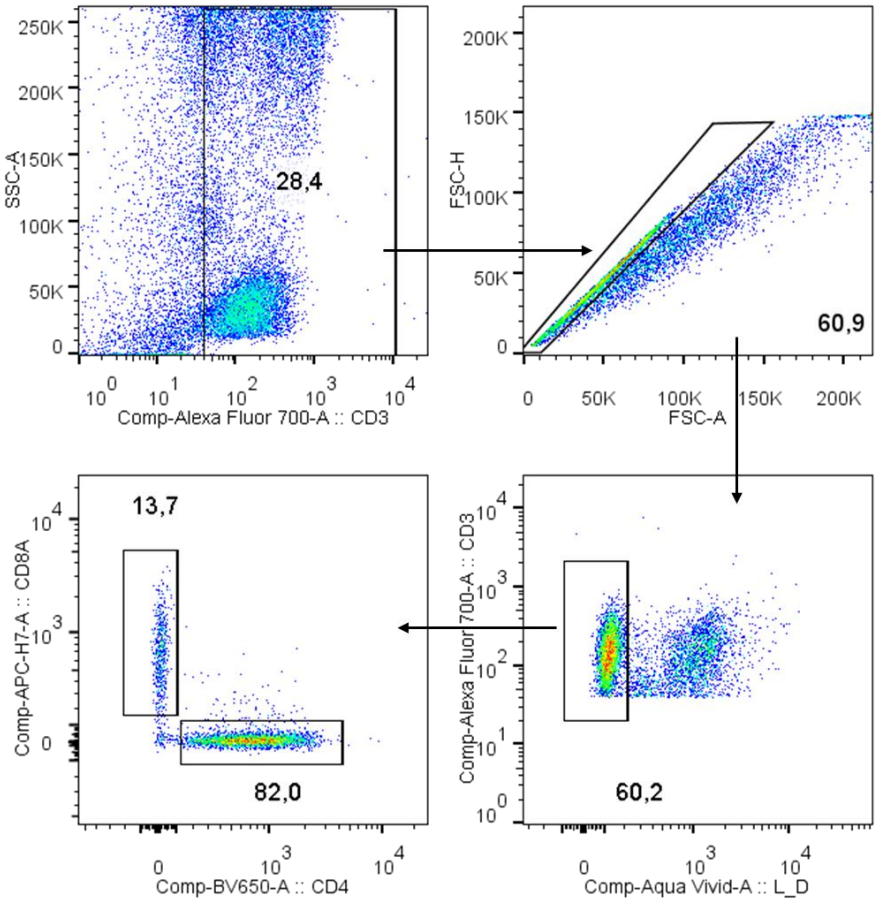
CD8 T cell phenotyping and staining ex vivo

Live/Dead Aqua Stain Kit (Thermo Fisher Scientific, Carlsbad, CA) was used to exclude dead CD3+CD4−CD8+ T cells from the analysis. All extracellular staining was performed in phosphate buffered saline, 2% fetal bovine serum, and 2 mM EDTA at 4°C for 1 h. After extracellular staining, the cells were washed and permeabilized using the BD Cytofix/Cytoperm Kit (BD Biosciences, Mississauga, ON, Canada). Permeabilized cells were incubated with the appropriate antibodies in a Perm/Wash buffer at 4°C for 1 h and stored in PBS until acquisition. Antibodies used for this group of participants are listed in Supplementary Table 3. A three-laser

BD Fortessa-X20 was used for acquisition, and results were analyzed by FlowJo software V10.0.7 (BD Biosciences). The gating strategy is shown in Supplementary Figure 1.

Supplementary Table 3: Antibodies used for measuring life CD4+ and CD8+ T-cell frequencies.

Antibody	**Fluorochrome**	**Clone**	**Compnay**
CD3	Alexa F 700	UCHT1	BD Pharmingen
CD4	BV650	L200	BD Horizon
CD8αα	APC/H7	SK1	BD Pharmingen
Live/Dead	Aqua vivid	-	Thermofisher



Supplementary Figure 1: Gating strategy used to measure CD8+ and CD4+ T-cell frequencies for additional participants added in Figure 21 in the main manuscript.