The Contribution of Tissue-Resident Alveolar Macrophages to HIV Persistence in Adults under Long-term Suppressive Antiretroviral Therapy

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Abstract

Background

While antiretroviral therapy (ART) has successfully reduced morbidity and mortality due to infection with human immunodeficiency virus (HIV), a cure remains elusive due to the obstacle of the latent viral reservoir. Persistence of HIV in particular cell subtypes, such as memory CD4+ T cells, and anatomical sites, such as the gut, lymph nodes, and brain, is well-documented. Since ART has become widely used, the lungs have been somewhat overlooked as a potential anatomical reservoir despite the elevated risk of pulmonary disorders among people living with HIV (PLWH) and features of the lung conducive to reservoir establishment, such as high cell density. Our group recently showed higher levels of HIV DNA in CD4+ T cells from bronchoalveolar lavage (BAL) fluid compared to CD4+ T cells from matched peripheral blood; however, in alveolar macrophages (AMs), the major cell type in BAL fluid, HIV DNA levels varied in a donor-dependent manner. AMs may be long-lived cells arising from an embryonic myeloid pre-cursor or derived from monocytes infiltrating from the periphery upon inflammation. Long-lived tissue-resident AMs of embryonic origin have been shown to possess the capacity of self-renewal without replenishment from peripheral monocytes. If these self-sustaining AMs prove to be latent cellular reservoirs of HIV, the implications for future cure approaches would be significant. As such, this project provides a comprehensive assessment of AMs to inform their role in viral persistence. This project also addresses the controversial question of macrophage permissiveness to direct HIV infection, which may also have implications in reservoir establishment.

Methods

Study participants were recruited from the Chronic Viral Illness Service (CVIS) at the McGill University Health Centre (MUHC) and underwent BAL. BAL fluid and matched blood were collected from each participant who were sorted into one of 4 cohorts based on their HIV and smoking status: HIV+ smoker, HIV+ non-smoker, HIV- smoker and HIV- non-smoker. HIV+ participants were successfully ART-treated with a minimum 3 years of undetectable viral load and CD4 count >350mm^3. All participants were free of pulmonary symptoms at time of bronchoscopy and did not suffer from chronic pulmonary issues. Flow cytometry was used to comprehensively characterize the immune phenotype of AMs by examining various markers of macrophage function, monocyte recruitment, proliferation, and potential origin. Due to the COVID-19

pandemic, participant recruitment was interrupted mid-way through this project and alternatives were sought to continue the work; some samples of cryopreserved BAL were used to complete the immunophenotyping data and a novel AM-like macrophage cell line 'Daisy' was characterized to establish its utility in macrophage research in the context of HIV. An *in vitro* infection protocol was established using monocyte-derived macrophages (MDMs) and Daisy cells with three strains of HIV to assess their permissiveness to direct infection. An R5-tropic NL4.3-Bal-IRES-HSA strain, X4-tropic NL4.3 strain and pseudotyped NL4.3-VSV strain were employed. This latter strain allowed for the bypassing of CD4 and co-receptor binding for entry of HIV as the virus is pseudotyped with the envelope of the vesicular stomatitis virus (VSV), which enters target cells by clathrin-mediated endocytosis.

Results

AMs displayed robust expression of phenotypic markers including CD206, HLA-DR, and CD169. In HIV+ individuals, AMs from smokers tended towards loss of CD163 expression, which could reflect the increased inflammation in the pulmonary milieu of these individuals. ART-treated HIV+ participants displayed a significantly higher frequency of CCR2-CD33+ AMs compared to HIV- people. Furthermore, these HIV+ donors showed a trend towards increased frequency of CX3CR1+ AMs over HIV- donors, suggesting there may be recently migrated monocytes from the blood into the lung mucosa. In both HIV+ and HIV- groups, smokers displayed the lowest frequency of CD71+ AMs, consistent with previous findings. CCR5+ AMs occur at a very low frequency suggesting they could be difficult to infect with HIV. Daisy cells expressed several macrophage markers, but only showed minimal expression of CD206 so could not be considered models of AMs specifically. Moreover, Daisy cells did not express the HIV-receptor CD4, so it was unsurprising that *in vitro* infection of Daisy cells with X4-tropic and R5-tropic strains was unsuccessful since these require CD4 binding for entry. Daisy cells were successfully infected with the pseudotyped NL4.3-VSV strain which gains entry to the cell by endocytosis. MDM controls were successfully infected with both NL4.3-Bal-HSA and NL4.3-VSV strains, but not the X4-tropic NL4.3 strain, which was expected given the low expression of CXCR4 in MDMs.

Conclusion

Even in the absence of pulmonary inflammation or clinical complications, PLWH under long-term suppressive ART may have increased frequency of infiltrating monocytes to the pulmonary

mucosa. Loss of CD163 and CD71 may be attributed to smoking. In contrast to MDMs, Daisy cells are not permissive to HIV infection using *in vivo*-like entry of CD4 binding. These findings add to our understanding of the interplay between pulmonary immunity and HIV persistence that will inform future macrophage reservoir study.

Résumé

Contexte

Alors que la thérapie antirétrovirale (TAR) a réussi à réduire la morbidité et la mortalité dues à l'infection par le virus de l'immunodéficience humaine (VIH), un remède curatif reste pour l'heure insaisissable en raison de l'obstacle du réservoir viral latent. La persistance du VIH dans des sous-types cellulaires particuliers, tels que les cellules T CD4+ mémoire, et des sites anatomiques, tels que l'intestin, les ganglions lymphatiques et le cerveau, est bien documentée. Depuis que la TAR est devenue largement utilisée, les poumons ont été quelque peu négligés en tant que réservoir anatomique potentiel malgré le risque élevé de troubles pulmonaires chez les personnes vivant avec le VIH (PVVIH) et les caractéristiques physiologiques des poumons, telle que leur densité cellulaire élevée, qui peuvent être propices à l'établissement de réservoirs. Notre équipe a récemment montré que les niveaux d'ADN du VIH sont, de façon consistante, plus élevés dans les cellules T CD4+ du liquide de lavage bronchoalvéolaire (LBA) par rapport aux cellules T CD4+ du sang périphérique apparié; alors que, dans le principal type de cellules présentes dans le LBA, les macrophages alvéolaires (MA), les niveaux d'ADN du VIH varient selon le donneur. Les MA sont des cellules à longue durée de vie qui proviennent soit d'un précurseur myéloïde embryonnaire ou peuvent dérivés de monocytes s'infiltrant depuis la périphérie lors de l'inflammation. Il a été démontré que les MA d'origine embryonnaire résidant dans les tissus à longue durée de vie possèdent la capacité d'auto-renouvèlement sans l'apport des monocytes périphériques pour leur reconstitution. Si ces cellules autosuffisantes s'avèrent être des réservoirs cellulaires latents du VIH, les implications pour les futures stratégies de guérison seraient très significatives. En tant que tel, ce projet se propose de fournir une évaluation complète du rôle des MA dans la persistance virale. Ce projet aborde également la question controversée de la permissivité des macrophages à l'infection directe par le VIH, qui peut également avoir des implications dans l'établissement des réservoirs.

Méthodes

Les participants ont été recrutés au Service des maladies virales chroniques (SMVC) du Centre universitaire de santé McGill (CUSM). Les participants ont fait don d'échantillons de sang, ont subi un prélèvement de LBA et ont été classés dans l'une des 4 cohortes en fonction de leur statut VIH et tabagique: fumeur VIH+, non-fumeur VIH+, fumeur VIH- et non-fumeur VIH-. Les

participants séropositifs recevaient une TAR efficace avec un minimum de 3 ans de charge virale indétectable (<20 copies/ml) et un taux de CD4> 350 mm^3. Tous les participants étaient exempts de symptômes pulmonaires au moment de la bronchoscopie et ne souffraient pas de problèmes pulmonaires chroniques.

La cytométrie de flux a été utilisée pour caractériser de manière exhaustive le phénotype immunitaire des MA en examinant divers marqueurs associés à la fonction des macrophages, leur recrutement, leur prolifération et leur origine potentielle issue des monocytes. En raison de la pandémie de COVID-19, le recrutement des participants a été interrompu à mi-chemin de ce projet et des alternatives ont été recherchées pour poursuivre le travail; certains échantillons de LBA cryoconservés ont été utilisés pour compléter les données d'immunophénotypage et une nouvelle lignée cellulaire de macrophages de type MA «*Daisy*» a été caractérisée pour établir son utilité dans la recherche sur les macrophages dans le contexte du VIH.

Un protocole d'infection *in vitro* a été établi en utilisant des macrophages dérivés de monocytes (MDM) des cellules Daisy avec trois souches de VIH pour évaluer leur permissivité à l'infection directe par le VIH. Une souche NL4.3-Bal-HSA à tropisme R5, une souche NL4.3 à tropisme X4, et une souche pseudo-typée NL4.3-VSV ont été employées. Cette dernière souche a permis de contourner la liaison des CD4 et des corécepteurs pour l'entrée du VIH, car le virus est pseudo-typé avec l'enveloppe du virus de la stomatite vésiculeuse (VSV), qui pénètre dans les cellules cibles par endocytose médiée par la clathrine.

Résultats

Les MA ont exprimé des taux élevés des marqueurs CD206, HLA-DR et CD169. Chez les personnes séropositives, les MA des fumeurs avaient tendance à perdre l'expression de CD163, ce qui pourrait refléter une inflammation accrue dans le milieu pulmonaire de ces personnes. Les personnes séropositives traitées par la TAR avaient une fréquence statistiquement plus élevée de MA CCR2-CD33+ par rapport aux personnes séronégatives. En outre, ces personnes séropositives semblaient également avoir une fréquence accrue de MA CX3CR1+. Ensemble, ces données suggèrent qu'il peut y avoir des monocytes ayant récemment migrés de la périphérie dans le pool de MA du LBA. Dans les groupes VIH+ et VIH-, les fumeurs affichaient la fréquence la plus faible de MA CD71+, similaire à ce qui a précédemment été montré. Les MA CCR5 + étaient faiblement représentés, ce qui suggère qu'ils pourraient être difficiles à infecter par le VIH. S'agissant des cellules Daisy, elles expriment certes plusieurs marqueurs caractéristiques des macrophages, mais

ne montrent qu'une expression minimale de CD206 et ne peuvent donc pas être considérées comme des modèles de MA spécifiques. En outre, les cellules Daisy n'expriment pas le récepteur du VIH CD4, il n'est donc pas surprenant que l'infection in vitro des cellules Daisy par des souches à tropisme X4 et à tropisme R5 n'ait pas été effective, car celles-ci nécessitent une liaison CD4 pour entrer dans les cellules. Les cellules Daisy ont été infectées avec succès par la souche NL4.3-VSV pseudotypée qui pénètre dans la cellule par endocytose sans utiliser la liaison au CD4. Les témoins MDM ont été infectés avec succès par les souches NL4.3-Bal-HSA et NL4.3-VSV, mais pas par la souche NL4.3 à tropisme X4, ce qui était attendu étant donné la faible expression de CXCR4 dans les MDM.

Conclusion

Même en l'absence d'inflammation pulmonaire ou de complications cliniques, les personnes séropositives traitées avec succès avec la TAR peuvent avoir une fréquence accrue d'infiltration de monocytes dans la muqueuse pulmonaire. La perte de CD163 et de CD71 peut être attribuée au tabagisme. Contrairement aux MDM, les cellules « *Daisy* » ne sont pas permissives à une infection par le VIH en utilisant une entrée de type CD4 in vitro. Ces résultats constituent un apport supplémentaire pour la compréhension de l'interaction entre l'immunité pulmonaire et la persistance du VIH qui éclairera les futures études sur les réservoirs du VIH au sein des macrophages.

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List of Abbreviations

AND (Fr.): Acide désoxyribonucléique
AIDS: Acquired immunodeficiency syndrome
AM: Alveolar macrophage
APOBEC: Apolipoprotein B mRNA editing catalytic polypeptide-like
ARDS: Acute respiratory distress syndrome
ART: Antiretroviral therapy
ARV: Antiretroviral (drug)
BAL: Bronchoalveolar lavage
BBB: Blood-brain barrier
bnAbs: Broadly neutralizing antibodies
CHUM: Centre hospitalier de l'Université de Montréal
COPD: Chronic obstructive pulmonary disease
COVID-19: Coronavirus disease of 2019
CSF: Cerebrospinal fluid
CTL: Cytotoxic T lymphocyte
CUSM (Fr.): Centre universitaire de santé McGill
CVIS: Chronic Viral Illness Service
DC: Dendritic cell
DN: Double negative (T cell)
DNA: Deoxyribonucleic acid
dNTP: deoxynucleotide triphosphate
EC: Elite controller
FBS: Foetal bovine serum
GALT: Gut-associated lymphoid tissue
HAART: Highly active antiretroviral therapy
HAND: HIV-associated neurocognitive disorder
HIV: Human immunodeficiency virus
HLA: Human leukocyte antigen
HSA: Heat stable antigen
HTLV: Human T-lymphotropic virus
IFITM: Interferon-induced transmembrane proteinIQR: Interquartile range
LBA (Fr.): lavage bronchoalvéolaire
LPS: Lipopolysaccharide
MA (Fr.): macrophages alvéolaires
MARCO: Macrophage receptor with collagenous structure
MDM: Monocyte-derived macrophage, macrophages dérivés de monocytes (Fr.)
MFI: Mean fluorescent intensity
MHC: Major histocompatibility complex
MRP: Multidrug resistance protein
MSM: Men who have sex with men
MUHC: McGill University Health Centre
NHP: Non-human primate
NNRTI: Non-nucleoside reverse transcriptase inhibitor
NRTI: Nucleoside reverse transcriptase inhibitor
OI: Opportunistic infection
PAMP: Pathogen-associated molecular pattern

PBMC: Peripheral blood mononuclear cell
PBS: Phosphate-buffered saline
PCP: *Pneumocystis* pneumonia
PEP: Post-exposure prophylaxis
PMA: Phorbol 12-myristate 13-acetate
PI: Protease inhibitor
PLWH: People living with HIV
PrEP: Pre-exposure prophylaxis
PRR: Pattern recognition receptor
PVVIH (Fr.): personnes vivant avec le VIH
QVOA: Quantitative viral outgrowth assay
RNA: Ribonucleic acid
SAMHD1: SAM and HD domain containing deoxynucleoside triphosphate triphosphohydrolase 1
SD: Standard deviation
SERINC: Serine incorporator protein
SIV: Simian immunodeficiency virus
SMVC (Fr.): service des maladies virales chroniques
t-SNE: t-distributed stochastic neighbor embedding
TAR (Fr.): thérapie antirétrovirale
TasP: Treatment as prevention
TB: Tuberculosis
UNAIDS: The Joint United Nations Programme on HIV/AIDS
VIH (Fr.): virus de l'immunodéficience humaine
VSV: Vesicular stomatitis virus, virus de la stomatite vésiculeuse (Fr.)
WHO: World Health Organization

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Preface

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

Author contribution for Chapter 1 Literature Review:
This chapter was written by Elaine Thomson, with advice and suggestions from Dr. Cecilia Costiniuk and Dr. Mohammad-Ali Jenabian.

Author contribution for Chapter 2 Methods:
The methods described in this chapter were designed by Elaine Thomson, Dr. Oussama Meziane, Edwin Caballero, Dr. Cecilia Costiniuk and Dr. Mohammad-Ali Jenabian.

Author contribution for Chapter 3 Results:
The experiments described in this chapter were performed by Elaine Thomson, Dr. Oussama Meziane, Edwin Caballero and Yulia Alexandrova.
Data analyses were performed by Elaine Thomson, Dr. Oussama Meziane, Dr. Cecilia Costiniuk, and Dr. Mohammad-Ali Jenabian.

Author contribution for Chapter 4 Discussion:
This chapter was written by Elaine Thomson, with advice and suggestions from Dr. Cecilia Costiniuk and Dr. Mohammad-Ali Jenabian.

Introduction

Rationale

Alveolar macrophages (AMs) are potential cellular reservoirs of HIV given their longevity and resistance to apoptosis. These cells are derived from one of two origins; embryonic or hematopoietic (monocyte-derived). The pool of embryonically-derived tissue-resident AMs can renew itself without the need for replenishment from circulating monocytes, so evaluating the origin of AMs becomes a key question in the context of their potential as a cellular HIV reservoir. Peripheral monocytes home to the lung mucosa and differentiate into macrophages in response to inflammation. Assessing the proportion of monocyte-derived AMs could provide insight into the pulmonary immune environment during antiretroviral therapy (ART)-suppressed HIV infection and smoking. Previous data from our group showed that cells from bronchoalveolar lavage (BAL) harbour more HIV DNA than do peripheral blood mononuclear cells (PBMCs). Furthermore, BAL CD4+ T cells specifically had 13-fold higher levels of HIV DNA compared to matched peripheral blood CD4+ T cells and cell-associated HIV RNA was detected in cells isolated from BAL. AMs are the predominant immune cell type present in the alveolar space, but when we assessed the HIV DNA levels in these cells, they varied greatly in a donor-dependent manner. To elucidate the potential role of these cells in HIV reservoir establishment and maintenance, this project seeks to investigate the immune phenotype of AMs and how it may vary based on the HIV and smoking status of the individual. In parallel, to address an unanswered question in the field of myeloid HIV research, this work will address the question of AM susceptibility to direct HIV infection *in vitro*.

Hypothesis

HIV infection and smoking cause a perturbation of the phenotypic profile of AMs which contributes to increased susceptibility of people living with HIV (PLWH) to pulmonary disorders. Long-lived, self-renewing AMs can be directly infected by HIV and contribute to viral persistence within the lungs of adults despite long-term suppressive ART.

Objectives

1. To perform phenotypic characterizations of AMs in HIV-infected *versus* uninfected donors and smokers versus non-smokers and assess these cells' embryonic *versus* peripheral developmental origin

2. To evaluate the permissiveness of AMs and monocyte-derived macrophages (MDMs) to direct HIV infection *in vitro*

Revised objectives due to the COVID-19 pandemic:

2a. To characterize and validate a recently described AM-like cell line, 'Daisy' and establish if they constitute a suitable model for HIV macrophage reservoir studies

2b. To infect Daisy cells and MDM controls *in vitro* to assess permissiveness to HIV infection as a potential model for *in vitro* AM infection.

Chapter 1: Literature Review

1.1 History of human immunodeficiency virus (HIV)

In the early 1980s a devastating disease was sweeping the globe and scientists were struggling to give the causative agent a name and a face[1]. In 2020 it took a matter of weeks to sequence the entire genome of the novel coronavirus SARS-CoV-2[2,3]), whereas it took years before the cause of Acquired Immunodeficiency Syndrome (AIDS) was identified as a single novel virus, and not another form of the retrovirus Human T-Lymphotropic Virus (HTLV), as was originally suspected. The causative agent was identified later in 1983 by Dr. Barre-Sinoussi/Dr. Luc Montagnier and Dr. Robert Charles Gallo as the lentivirus now known as Human Immunodeficiency Virus (HIV)[4,5]. Further research throughout the past four decades has filled in the picture of the origin and evolution of HIV. We now know that HIV is a zoonotic disease which entered the human population via several mutations of Simian Immunodeficiency Viruses (SIVs) infecting non-human primates[6]. The genetic diversity if HIV-1 is large, and sequences are divided into groups, designated M (major), N (new), O (outlier), and P, to continue the alphabetical order[7,8]. Group M accounts for the majority of HIV-1 infections today, and is further subdivided into 9 clades, named 'A' through 'I', with Clade B accounting for most infections in Europe and North America, and Clade C being much more prevalent in southern Africa[7]. HIV-1 Group M zoonotic transmission occurred through a chimpanzee species in central Africa infected with SIVcpz[9]. It is important to note that there is a less common form of the virus, HIV-2, which also infects humans and can cause AIDS, but is predominantly localized to the West African region as opposed to the global distribution of HIV-1[10]. HIV-2 entered the human population during another separate transmission event from a different SIV which infects sooty mangabeys in nature, SIVsm[11]. Due to their genetic diversity, certain diagnostic tests and antiretroviral drugs (ARVs) for HIV-1 will not be effective for HIV-2[12,13]. Compared to HIV-1, HIV-2 is less transmittable and disease progresses more slowly[14]. Various factors contribute to a generally more effective immune response to HIV-2 and lower mortality rates as a result[10,15].

1.2 Epidemiology of HIV Infection

While the HIV research field has made great strides in the nearly four decades since its beginning, the burden of this virus remains significant. There were ~38 million people living with HIV (PLWH) worldwide at the end of 2019, 1.7 million of which were new infections[16] (Fig. 1). In Canada ~62,000 people were infected with HIV at the end of 2018[17]. Despite the existence of very effective HIV prevention strategies, ~2,100 new HIV infections occur in Canada annually[18.] In

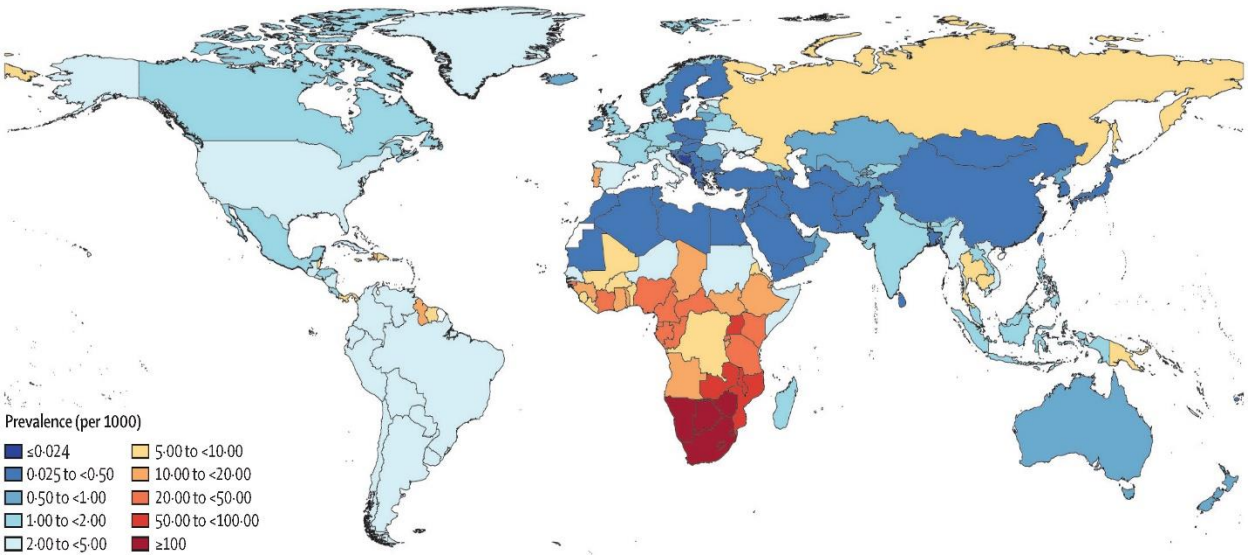

Figure 1. Prevalence of HIV worldwide. 2017 data for incidence per 1000 people of HIV infection[23].

recent years, improvement of ARVs has meant that an HIV diagnosis is no longer a death sentence as it once was. Despite its efficacy, however, antiretroviral therapy (ART) is a life-long requirement, which is estimated to cost on the order of $1.3 million in Canada.[19] In resource-poor areas, adherence to treatment may be even more challenging due to issues with infrastructure and periodic stock shortages, which is detrimental to treatment success[20]. Despite substantial advances in therapies, parts of the world such as southern Africa still see vast generalized prevalence of HIV[16]. Furthermore, in certain areas such as the Middle East and parts of Asia, new infections are actually on the rise[21]. Many factors contribute to the struggle to control the virus spread, such as stigma surrounding diagnosis, inefficient access to testing and treatment, as well as unstable socioeconomic conditions. In 2015 The Joint United Nations Programme on HIV/AIDS (UNAIDS) set the 90-90-90 goal for HIV in 2020; 90% of people with HIV know their status, 90% of those people are on antiretroviral treatment and 90% of those have a suppressed viral load[22]. It is clear at the end of 2020 that we have not reached this goal, as was predicted by the forecast at the end of 2019[23] (Fig. 2). Even if these milestones are eventually reached, however, the economic burden of lifetime treatment of ARVs, not to mention the non-AIDS co-morbidities seen in PLWH make the search for a cure just as relevant today as thirty years ago.

Furthermore, ART does not restore normal immune status[24]. PLWH are still at risk for several non-AIDS co-morbidities, even with effective ART treatment[25]. As parts of the HIV+ population are now starting to reach elderly ages, particular risks have come to light, especially cardiovascular

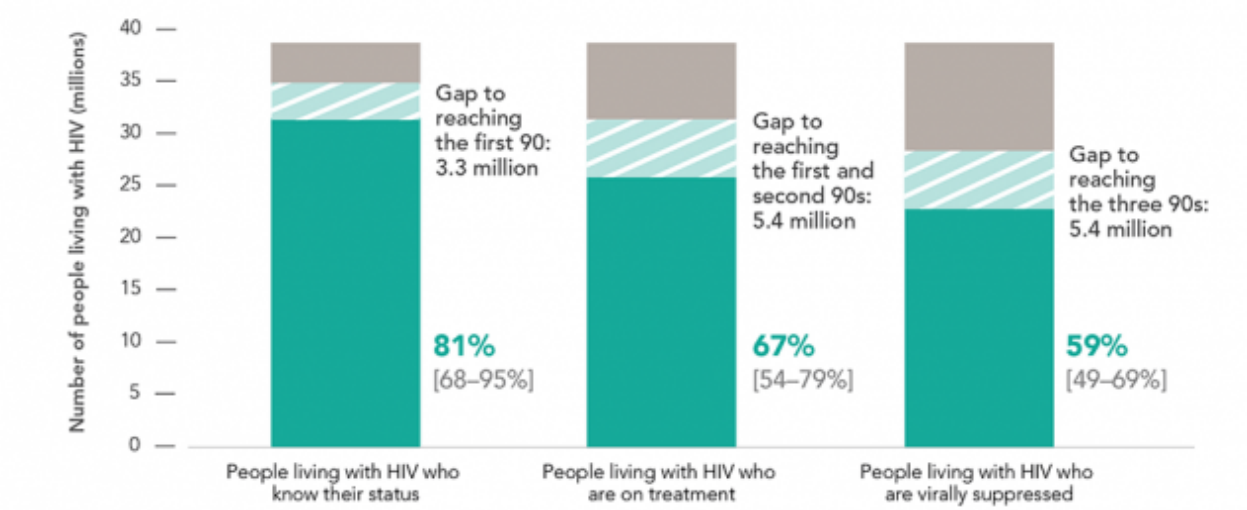

Figure 2. UNAIDS 90-90-90 targets. Figures showing actual figures compared to 90% target for proportion of PLWH globally who know their status, of whom who are on ART, of whom who are virally suppressed[22].

disease, metabolic disease and neurocognitive disorders[24,26]. PLWH are also at higher risk than uninfected individuals for non-AIDS associated malignancies[27]. As alluded to previously, ARVs have secondary effects, such as reduced bone mineral density and renal toxicity that can take a toll over time, though ARVs currently on the market exhibit significantly less toxicity than early ARVs[27-29]. Of course, these complications could be eliminated all together with the advent of an HIV cure.

1.3 Clinical phases of HIV Infection and AIDS

The acute phase of HIV is characterized by a flu-like or mononucleosis-like illness, with fevers, chills, sweats, sore throat and myalgias. During this phase, when HIV is disseminated throughout the body, many individuals do not know they have become infected with HIV[30]. This is followed by a period of clinical latency for about 7-10 years, when persons are asymptomatic[30,31] (Fig. 3). The rate at which individuals progress vary based on both viral and host immune factors[30]. When HIV infection goes untreated, the disease natural history is characterized by depletion of the CD4+ T cells which are the target of the virus[30]. Since CD4+ T 'helper' cells are known as the master regulators of the immune system, it follows that the result is immune dysfunction. One characteristic effect of acute HIV infection is the 'leaky gut'; infection of the gut-associated lymphoid tissue (GALT) is associated with significant microbial translocation out of the gut mucosa[32]. The GALT accounts for 40-60% of the total T cell population in the body which is

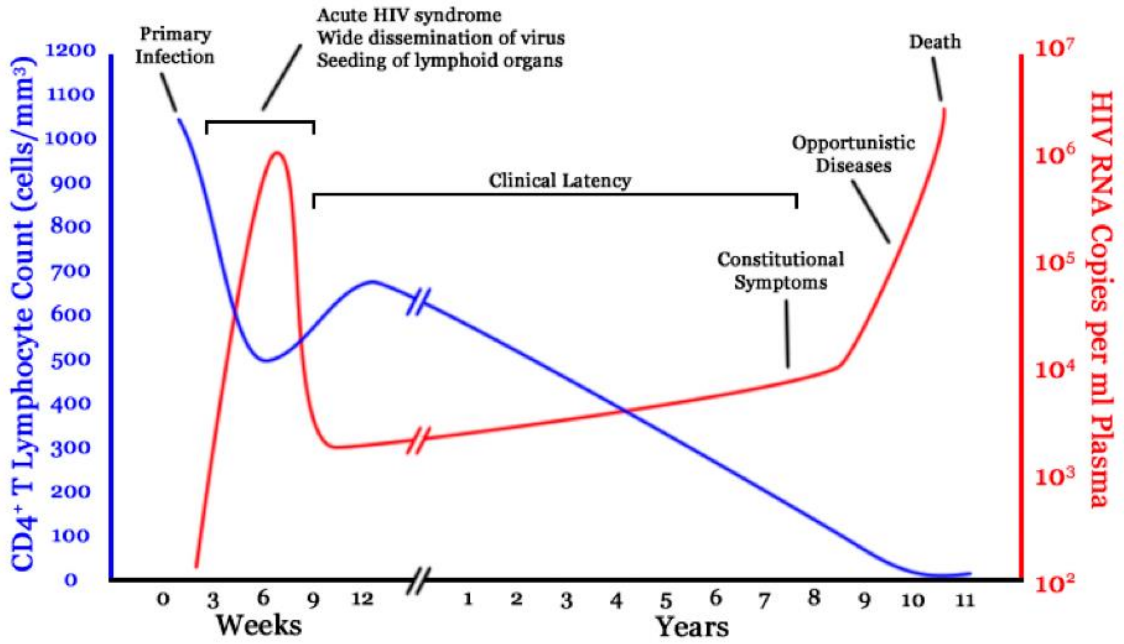

Figure 3. Natural history of HIV. In untreated infection, CD4+ T cell depletion is coupled with increased in viral load in an inversely proportional manner[31].

depleted during acute HIV infection[33] and this, in turn, compromises the integrity of the intestinal barrier, leading to microbial translocation to the periphery[34]. Leakage of bacteria leads to chronic systemic inflammation due to the resulting increase in plasma lipopolysaccharide (LPS) levels[35]. Eventually, there is progressive CD4+ T cell decline over years and the immune system becomes unable to fight off opportunistic infections (OIs) such as *Mycobacterium avium intracellulare* and *Pneumocystis* pneumonia (PCP)[36]. Development of OIs and depletion of CD4+ T cells to below 200/mm^3 of blood are designations for the onset of AIDS[37]. This depletion may take many years as shown in Fig. 3 and is complemented by a steady rise in HIV burden in the blood. This chronic inflammation leaves the individual susceptible to many co-morbidities, such as cardiovascular disease and chronic obstructive pulmonary disease (COPD), even with successful treatment[24,26].

1.4 HIV Life Cycle

HIV-1 is a positive sense single-stranded RNA virus whose genome consists of 9 genes; structural genes (*gag*, *pol*, *env*), regulatory genes (*tat*, *rev*) and accessory genes (*vif*, *vpr*, *vpu*, *nef*)[38] (Fig. 4). *Gag* encodes the matrix, capsid (p24) and nucleocapsid proteins; *pol* encodes the protease, reverse transcriptase and integrase proteins; while *env* encodes the surface (gp120) and transmembrane (gp41) proteins. These proteins are the main targets of current HIV vaccine designs[39]A signature

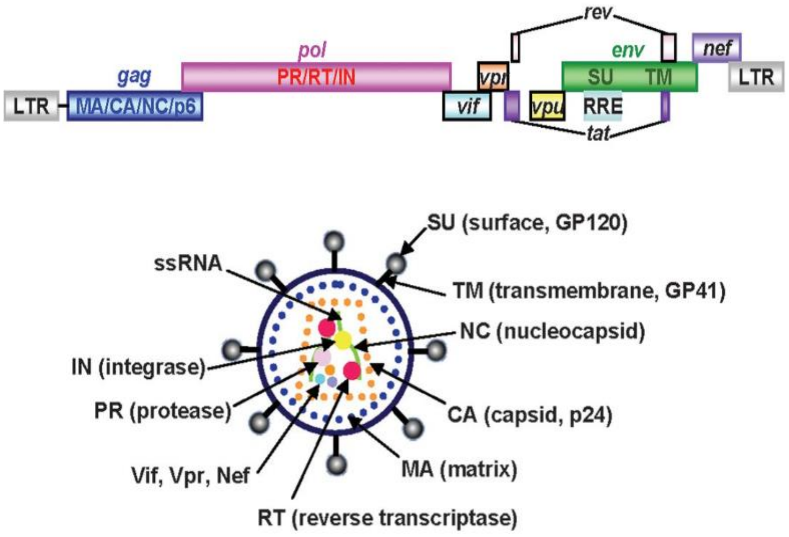

Figure 4. HIV genome and structure. Overview of HIV-1 genome (top) and assembled structure of proteins and enzymes (bottom)[39].

feature of retroviruses, including HIV, is their ability to integrate their genome into that of the host using their integrase protein. Indeed, this feature has been exploited using attenuated retroviruses for gene editing technologies in recent years. The genome of HIV-2 is slightly different, and importantly contains the accessory protein gene *vpx* but not *vpu*[40] (Fig. 5). This has important implications for the infection of certain cells such as macrophages as Vpx disrupts the function of the host restriction factor SAM domain and HD domain-containing protein 1 (SAMHD1), described below[41]. These various crucial components work in tandem, allowing HIV to complete its life cycle and produce more virions to go on and infect additional target cells.

1.4.1 Viral entry

Entry of the HIV-1 virion into a target cell is mediated by binding to CD4 on the cell surface by the viral envelope surface protein gp120. Following binding, a conformational change in gp120 exposes binding sites for co-receptors. The principal co-receptors for HIV are the CCR5 and CXCR4 chemokine receptors, however, other chemokine receptors have also been shown to perform this function, less usually[42]. Fusion can be blocked by host interferon-induced transmembrane proteins (IFITMs)[43]. Following this fusion step, the virus is internalized and enters the cytoplasm. Reverse transcription occurs, generating a DNA copy of the viral genome from the positive sense single stranded RNA virus[38]. This DNA is then translocated to the nucleus where viral integrase protein allows integration of the DNA into the host cell genome[38].

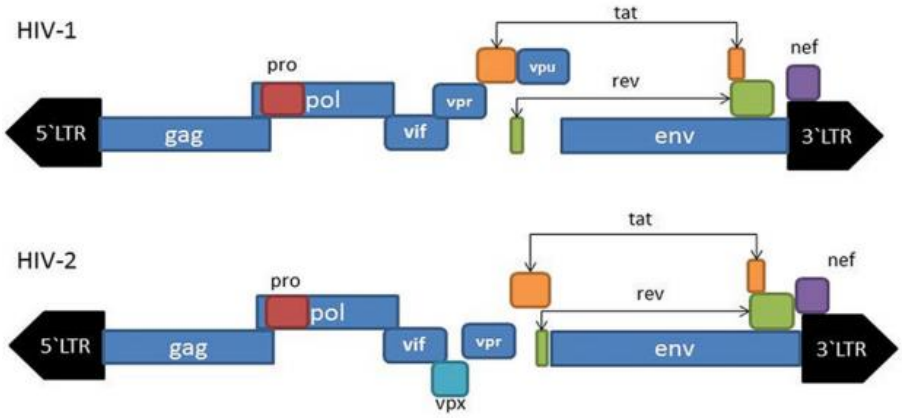

Figure 5. Comparison of the genomic organizations of HIV-1 and HIV-2. HIV-1 contains the accessory protein gene *vpu* while HIV-2 contains *vpx*[40].

1.4.2 Viral integration

A key attribute of retroviruses is their ability to integrate their genome into that of the host target cell. This is accomplished using integrase, an enzyme encoded by the *pol* gene[38]. While studies have shown that the integration sites are not entirely random, there is much variability in where the site may fall in the host DNA[44]. This integration also allows for the establishment of a latent viral reservoir in quiescent cells. Certain conditions favour integration of the HIV genome without lysing the host cell, such as a lack of deoxynucleoside triphosphates (dNTPs) in the cell for use by reverse transcriptase. This is the case in cells such as macrophages which express the host restriction factor SAMHD1 which depletes the cellular dNTP pool[45]. Interestingly, it has been shown that the activation state of SAMHD1 is responsible for a disparity in HIV-1 susceptibility based on biological sex[46].

1.4.3 Viral replication

Once integrated into the host genome, viral DNA is transcribed by the host cellular machinery and immature viral polyproteins are produced from the *gag*, *pol*, and *env* genes[47]. A single HIV polyprotein is initially translated which is then cleaved by a protease enzyme to generate mature functional proteins, including capsid proteins such as p24; replication proteins such as reverse transcriptase, integrase and protease; and the surface proteins gp120 and gp41[47]. Various accessory proteins, including Nef, Vif and Vpu are also encoded, which aid the virus to evade elements of the immune system and are responsible for some of the cellular hallmarks of HIV infection,

including down-regulation of CD4 and major histocompatibility complex (MHC) class I molecules by Nef[48,49].

1.4.4 Viral particle packaging

Once viral RNA and proteins are generated, they are packaged into new virions which bud at the cell surface[47]. The p24 capsid protein detectable by antibody is often used for infection, testing and detection methods such as western blot[50]. Another host restriction factor the virus must contend with at this stage are the apolipoprotein B mRNA editing catalytic polypeptide-like proteins (APOBECs)[51]. These are incorporated into the budding viral particle and introduce mutations into the viral RNA[51]. However, the HIV accessory protein Vif can counteract APOBEC function[51]. Furthermore, other restriction factors may disrupt physical processes, such as tetherins which prevent budding of the viral particle and serine incorporator proteins (SERINCs) which are incorporated into the viral particle and prevent its fusion to the next target cell[52,53]. SERINCs are also downregulated by Nef, however, and this is a perfect example of the co-evolution of viral accessory proteins and immune restriction factors[54].

The completion of the viral replication cycle is an ideal case for the HIV virion, and only occurs if conditions are optimal and the virus is able to circumvent the many host restriction factors. As such, the cycle is only completed in metabolically active CD4+ T cells. If the virus infects a resting CD4+ T cell which is more quiescent, viral replication may be blocked either pre- or post-integration, depending on such factors as dNTP availability for reverse transcription. Resting CD4+ T cells with low levels of dNTPs are ideal candidates for the establishment of viral latency, therefore; indeed it is the transitional and central memory CD4+ T cells which are more likely to become reservoir cells[55]. HIV can also infect other cells such as macrophages and dendritic cells (DCs) by different entry pathways such as phagocytosis and micropinocytosis, so virus may be internalized without replicating and can then be passed on to a CD4+ T cell such as via the immunological synapse[56].

1.5 Antiretroviral therapy

The rapid and rigorous research into ARVs has spearheaded the shift of HIV from a fatal to a chronic illness for many of those diagnosed. Early iterations of ARVs quickly failed due to the ability of HIV to rapidly mutate and develop resistance[57]. As such, the advent of combination therapy or highly active antiretroviral therapy (HAART) proved a necessary step to overcome this

adaptability of the virus. HAART involves prescribing a cocktail of ARVs, with the presumption that while some strains may be able to adapt resistance to one drug, it is highly unlikely that a single strain will form resistance to multiple drugs. Complementary to the various stages of the HIV life cycle, the different classes of available ARVs disrupt distinct stages depending on their mode of action[58,59] (Fig. 6). Entry inhibitors prevent internalization of HIV by the target cell, nucleoside reverse transcriptase inhibitors (NRTIs) and non- nucleoside reverse transcriptase inhibitors (NNRTIs) prevent effective reverse transcription of HIV DNA, integration inhibitors prevent insertion of HIV DNA into the host genome, and protease inhibitors (PIs) prevent cleavage and resulting activation of viral proteins from the translated polyprotein[58]. Current HAART comprises two NRTIs and one drug from another class; an NNRTI, integrase inhibitor or PI[60].

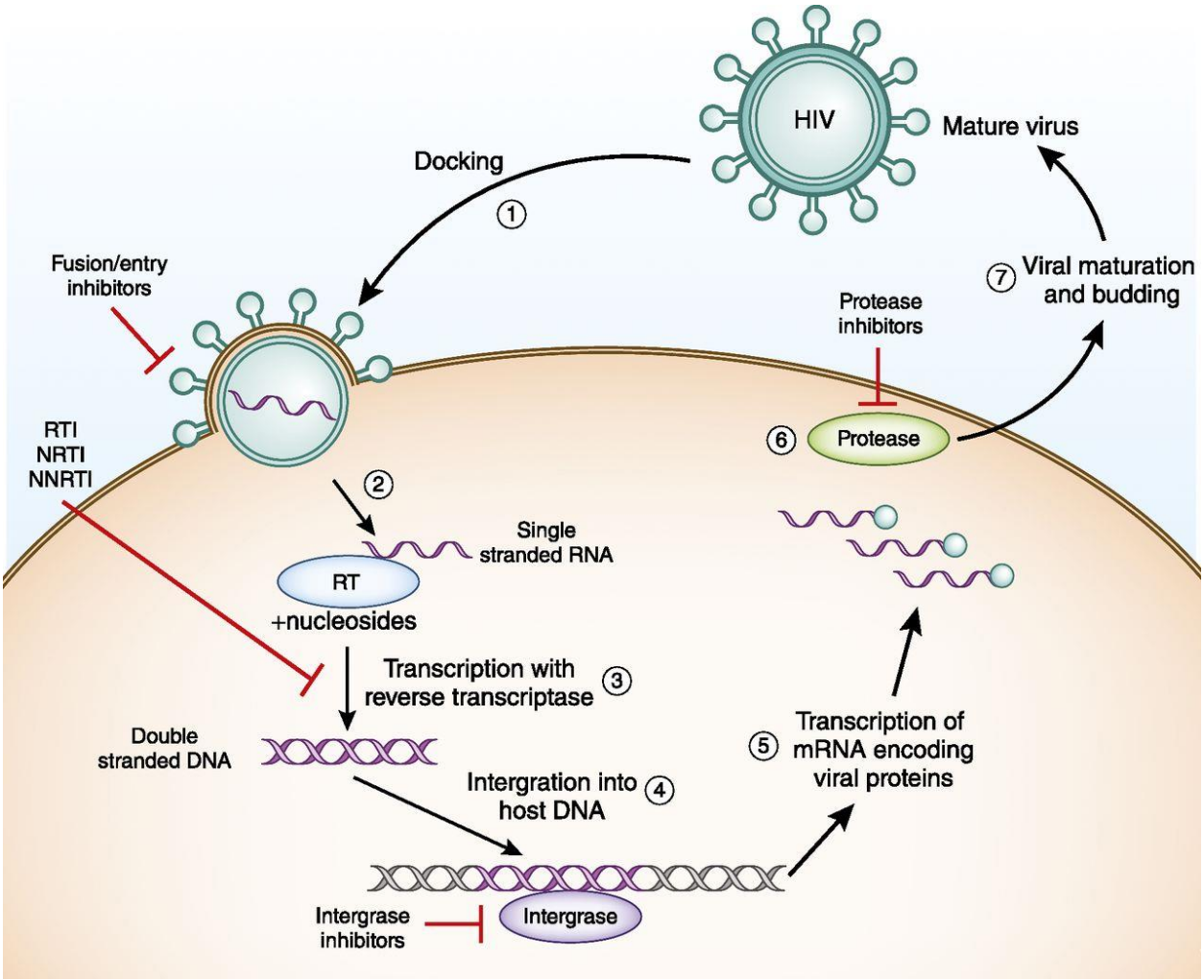

Figure 6. Life cycle of HIV and ARV classes which inhibit them. Various stages of the HIV life cycle are targeted by different classes of ARVs. RT: reverse transcriptase RTI: RT inhibitors, NRTI: nucleoside RT inhibitors, NNRTI: non- nucleoside RT inhibitors[59].

Many of these cocktails are administered in a single-tablet format, but clinical trials are ongoing for long-acting injectables [61] which would only need administering monthly; these are expected to be available on the Canadian market in the near future. The medical community is constantly updating the recommendations for these drugs in terms of the benefits for the patients based on their particular case, taking into account sex, age, lifestyle, pregnancy, and a host of other factors[60]. As the side effects of these drugs became more tolerable, the benefits expanded beyond PLWH.

1.6 ARVs as prophylactics

ARVs are now used in other applications beyond ART; the resounding success of pre-exposure prophylaxis (PrEP) is perhaps the most publicized. PrEP is a preventative treatment whereby individuals in certain groups, such as men who have sex with men (MSM), take ARVs in anticipation of potential exposure to HIV, either as a daily regimen or even only on the specific day they are engaging in higher risk activities[62,63]. Post-exposure prophylaxis (PEP) is a similar concept to PrEP, but the ARVs are taken after a possible exposure to prevent establishment of HIV infection, though this has to be done within hours or days of exposure[62]. Furthermore, ARVs are used for treatment as prevention (TasP), where by controlling plasma viral load in the seropositive partner, the seronegative partner in a serodiscordant couple is protected from becoming infected[62]. The efficacy of ARVs and implementations such as TasP is exemplified by the Public Health Agency of Canada statement in 2017 that "undetectable = untransmittable", indicating that PLWH who maintain an undetectable plasma viral load with ART for at least 6 months have effectively no risk of transmitting HIV through sexual route of transmission[64].

1.7 Obstacles to vaccine development

The field of HIV vaccine research is still thriving but is challenged by the elusive and adaptive nature of the virus, which has an extremely rapid mutation rate. This is primarily due to the error-prone reverse transcriptase and short generation time that characterize HIV[65]. Efforts are complicated by the fact that HIV targets the very cells which are the 'master regulators' of the immune system, CD4+ T helper cells. Furthermore, the main premise of most modern vaccine development strategies is to 'preview' the body to prime the natural immune response that occurs upon contact with the infectious agent, but in the case of HIV the natural response is ultimately ineffective, so it is not enough to simply encourage this mechanism to occur more rapidly. The majority of current research focuses on one of two approaches: generating antibodies to HIV or

targeting CD8+ cytotoxic T lymphocytes (CTLs) to HIV-infected cells[66]. Broadly neutralizing antibodies (bnAbs) have become an area of interest because of the high levels of these antibodies in elite controllers (ECs), a small proportion of individuals who are HIV+ but are able to control the infection without the use of ARVs[67]. T cell approaches seek to improve the immune system's reaction to the infection, and studies in non-human primates (NHPs) have shown promising results which suggest the answer may lie in the human-leukocyte antigen (HLA) type employed by the T cells[68]. Trials are constantly ongoing but so far the only case which stands out is that of the RV144 trial from Thailand which showed ~30% protection in a large cohort of individuals at community risk[69]. So for the time being, the search for a safe and effective vaccine continues.

1.8 Latent HIV reservoirs: barrier to a cure

Despite the widespread success of ART and the ability to achieve a state of undetectable levels of viral RNA in the periphery, there is still no cure for HIV due to viral reservoirs. These cell populations harbour replication-competent virus despite ART[70]. In some privileged anatomical sites, such as the central nervous system, ongoing viral replication may occur with ART due to insufficient penetration of ARVs[71]. Establishment of latent HIV reservoirs occurs when cells are infected with virus which is integrated into the host genome but then ceases to replicate and remains dormant in these cells[72]. However, following appropriate antigenic stimulation, viral production may be reactivated. So, while patients may appear virally suppressed while on ART, and only 1/10,000 peripheral CD4+ T cells contain HIV DNA[73], the viral load rebounds once treatment is interrupted[74]. SIV infection studies on NHPs have exemplified the fact that viral reservoirs are seeded almost immediately after infection in various cell types and anatomical sites as elaborated below[75].

1.9 Cellular reservoirs of HIV: memory CD4+ T cells *versus* tissue-resident macrophages

The best-characterized cellular reservoir is the resting CD4+ T cell, specifically the memory subset whose more quiescent nature lends itself to viral latency[55,76,77] (Fig. 7). Despite recent interest in cellular markers to identify reservoir cells such as CD32a, this could not be validated and there remains no definitive indicator of reservoir cells[78,79]. Certain CD4+ T cell subsets have been identified as particularly strong reservoir candidates, including transitional and effector memory CD4+ T cells (T_{TM} and T_{EM}) and T follicular helper cells (T_{FH}), as well as CCR6+ and CXCR3+ CD4+ T cells[55,80-82].

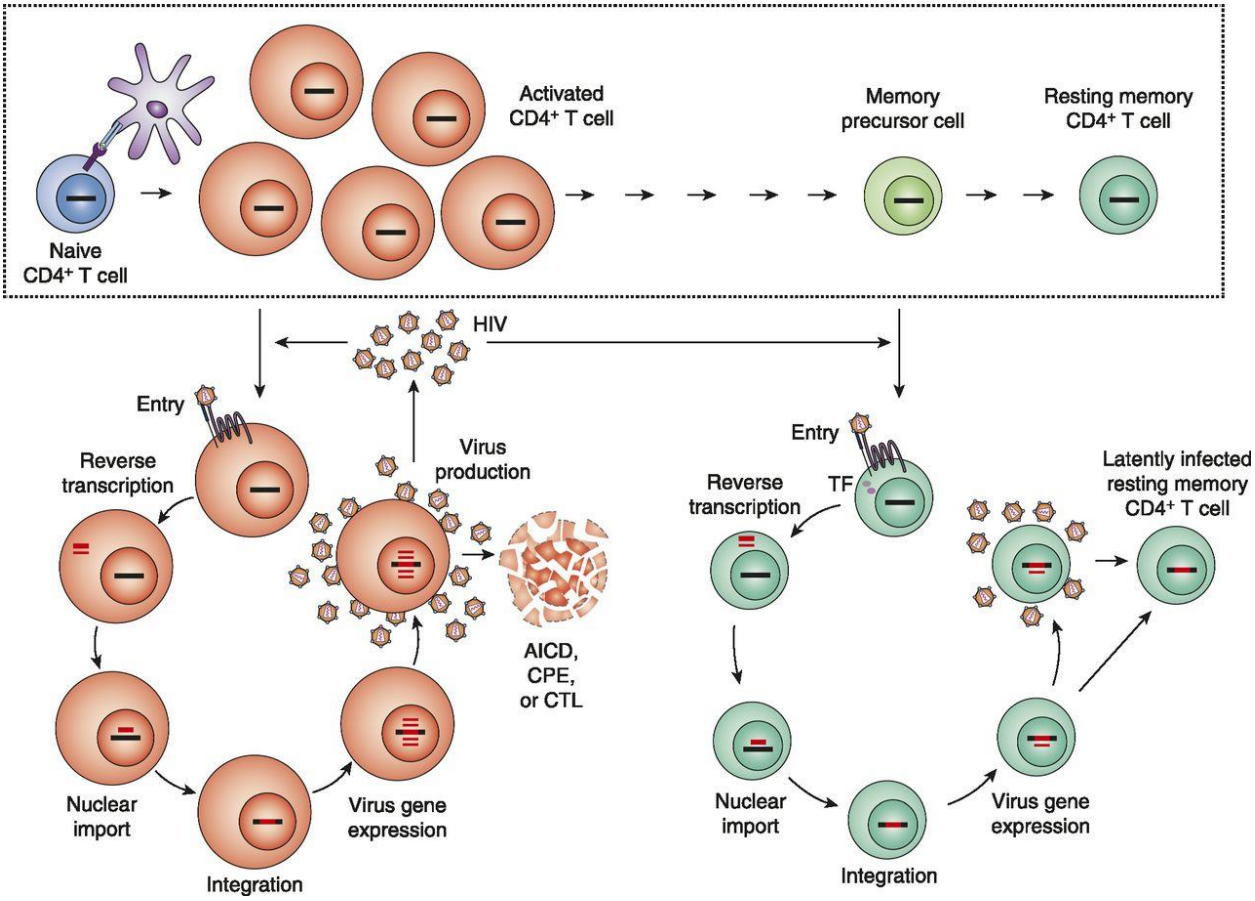

Figure 7. CD4+ memory T cell reservoir establishment. Rather than lysing to release new HIV virions, quiescent CD4+ memory T cells may instead remain intact and become a latent reservoir[76].

Myeloid cells can also act as cellular HIV reservoirs. In untreated HIV infection, monocytes are infected, and these may differentiate into macrophages[83], and macrophages appear to become targets of infection upon CD4+ T cell depletion[84]. Humanized mouse models have shown that HIV infection can be established and maintained exclusively in myeloid cells and that mature bone marrow and spleen macrophages act as viral reservoirs[85,86]. Non-human primate models have also shown productive infection of spleen, lymph node, brain and lung tissue macrophages *in vivo* with SIV using a quantitative viral outgrowth assay (QVOA) adapted to tissue macrophages which are limited in number[87]. However, while splenic myeloid cells from ARV-treated rhesus macaques harboured SIV DNA, replication-competent virus could not be detected in these cells, despite showing this in ART-naive animals[88]. In humans, a recent study by Ganor *et al.*[89] showed that replication-competent virus was present in urethral macrophages. Conversely, myeloid cells in the colon of ART-suppressed individuals could not be designated as a viral reservoir[90]. Furthermore,

a recent study demonstrated inducible detection of HIV DNA under suppressive ART in human liver macrophages, though the researchers concluded they could not be classified as a cellular reservoir since HIV RNA was not detectable[91]. The diversity amongst these studies demonstrates the importance of investigating tissue-resident macrophages as separate populations based on their niche.

1.10 Anatomical reservoirs of HIV

Latent HIV reservoirs may be found in various anatomical sites such as the central nervous system, gut and lymphatic tissues[92,93] (Fig. 8). However, the physiology and immunological environment

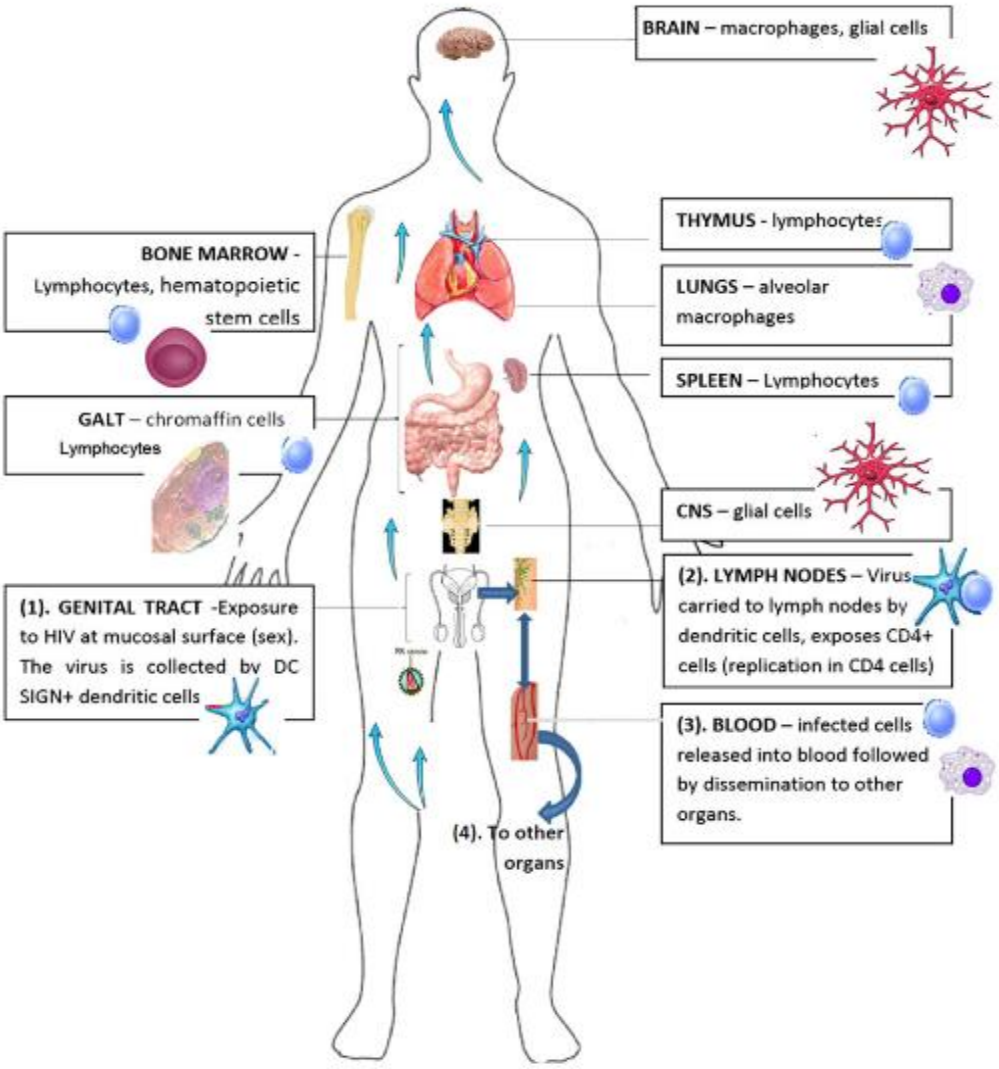

Figure 8. Anatomical reservoirs of HIV. Latent HIV reservoirs are found in various anatomical sites throughout the body[93].

vary greatly between these sites. Due to the common route of infection in adults by sexual transmission[94], the genital tract is an obvious candidate for HIV reservoir establishment. Indeed, vaginal secretions show varying levels of viral shedding even under ART[95]. HIV reservoirs have also been identified in the secondary lymphoid tissues, such as the spleen and lymph nodes[92].

The brain and testes are both immune privileged sites with a physical barrier preventing the infiltration of CTLs and ARVs[96,97]. The blood-brain barrier (BBB) defines the immune privilege of the brain, protecting the brain from harmful substances, autoimmune disorders and infections[98]. Despite this, HIV is detected in cerebrospinal fluid (CSF) as well as perivascular macrophages and microglia in infected individuals[99,100]. HIV is able to bypass the BBB, however, using a 'Trojan horse' method whereby the virus infects monocytes in the periphery but does not replicate until regular turnover of perivascular macrophages recruits monocytes to the brain to differentiate, thereby escaping immune detection[101]. Neurological conditions associated with HIV have been collectively named 'HIV-associated neurocognitive disorder' or HAND, and are present in nearly half of successfully ART-treated individuals[102]. Similarly, in the testes, breach of the blood-testis barrier, such as by sperm, results in an immune response to keep this tissue isolated and protected[103]. During ART-treated HIV infection, a small number of T cells harbouring HIV DNA were identified in testicular tissue samples of five out of six individuals[97].

Conversely, the lungs are consistently exposed to the external environment and its various stimuli, making it a non-lymphoid immunological effector tissue, similar to the GALT described above. In bronchoalveolar lavage (BAL) samples which represent the lung mucosal environment, HIV DNA can be found both in the CD4+ T cells and alveolar macrophages (AMs)[104]. A myeloid-only humanized mouse model also demonstrated that HIV DNA could be found in the lung during suppressive ART[85]. AMs are equipped with p-glycoprotein and multidrug resistance protein (MRP) efflux pumps, which can eject some ARVs, and reduce their efficacy[105-107]. As such, the lungs warrant further investigation as various anatomical sites must be treated as independent to understand reservoir maintenance and potential HIV eradication strategies.

1.11 Ontogeny of tissue-resident macrophages

Macrophages are integral players in the immune system whose key functions include clearing pathogens and dying cells by phagocytosis and efferocytosis; assisting with the maintenance of tissue homeostasis; engaging the innate immune system during inflammatory responses; and playing a role in tissue repair[108]. However, the comprehensive role of tissue-resident macrophages

is much more nuanced and, indeed, specific to the tissue microenvironment in which they are found. As Bleriot *et al.*[109] described in their recent review of tissue-resident macrophages, the identity of these cells is a reflection of their ontogeny, anatomical niche, adaptive age and inflammation status. While it has been understood for decades that monocytes are the precursors of macrophages and differentiate in response to inflammation[110], it has recently become clear that other macrophage precursors are present even at embryonic stages of development[111] (Fig. 9). Intricate fate-mapping studies in mice have demonstrated that these progenitor cells are not sourced from the bone marrow but are separate macrophage progenitors found in the yolk sac and foetal liver[112]. Furthermore, this pool of tissue-resident macrophages has been shown to replenish itself without contribution from circulating monocytes[113]. This model has been explored in humans, such as in the context of lung transplant, with HLA-mismatching or sex-mismatching used to identify whether AMs are of donor or recipient origin, with mixed results[114-116]. These discoveries have broadened our understanding of the interplay between monocytes and macrophages in homeostasis and immunity and has highlighted the significance of the

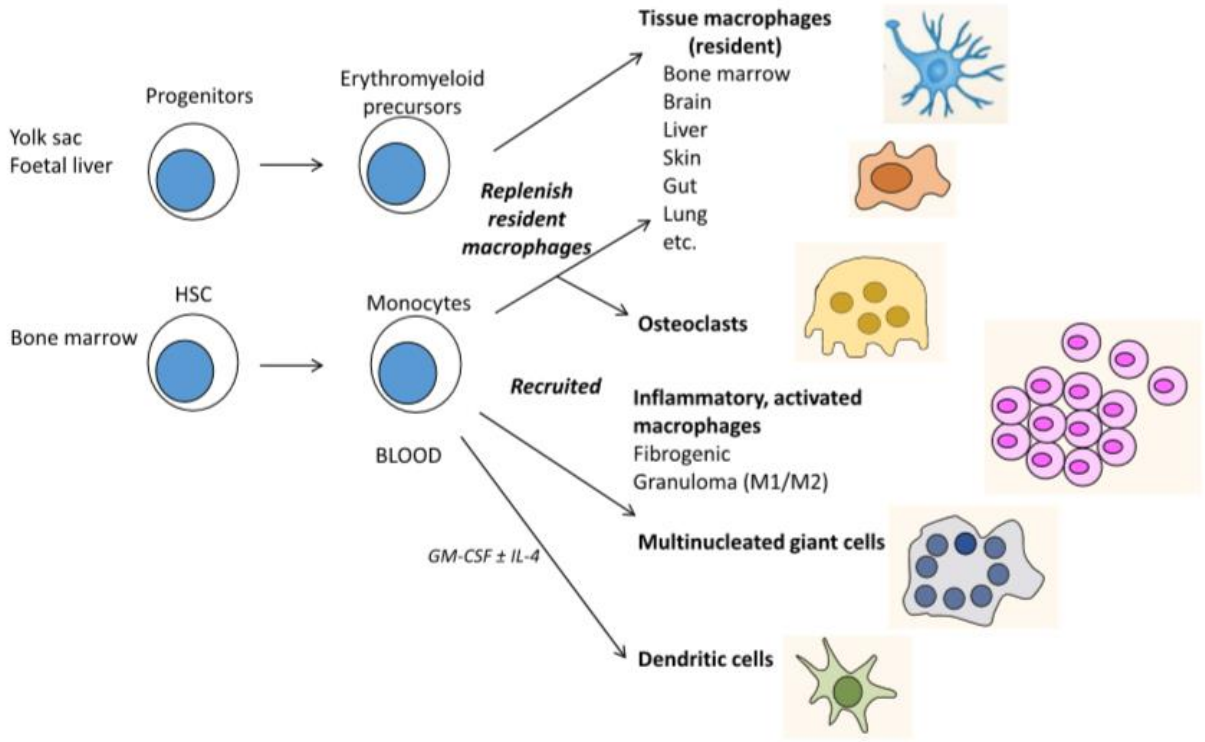

Figure 9. Ontogeny of tissue macrophages. Tissue macrophages may be derived from myeloid precursors originating in the yolk sac or foetal liver, or derived from monocytes infiltrating from the periphery[111].

environment of these tissue-resident macrophages. Indeed, macrophages express a myriad of proteins to perform their functions, such as cytokine and chemokine receptors, pattern recognition receptors (PRRs), and antibody receptors (Fig. 10). The levels and combinations of these markers, however, are entirely dependent on the niche in which they inhabit. For example, the lung mucosa is consistently exposed to stimuli from the exterior, so the AMs populating this tissue must perform sentinel duties to neutralize pathogens they may encounter, while simultaneously playing an anti-inflammatory role so the pulmonary space is not in a constant state of inflammation[108,117]. Historically, one way of characterizing the behaviour of macrophages has been using the M1/M2 designation, whereby macrophages are described as 'polarizing' to either an M1 pro-inflammatory phenotype and expressing cytokines such as TNF-α, or an M2 phenotype which is more quiescent, expressing IL-10 and other cytokines associated with anti-inflammatory pathways[118]. The reality is much more complex than this simplified model, as macrophages often express or secrete proteins associated with both M1 and M2 polarization; indeed, these phenotypes may be seen as two ends

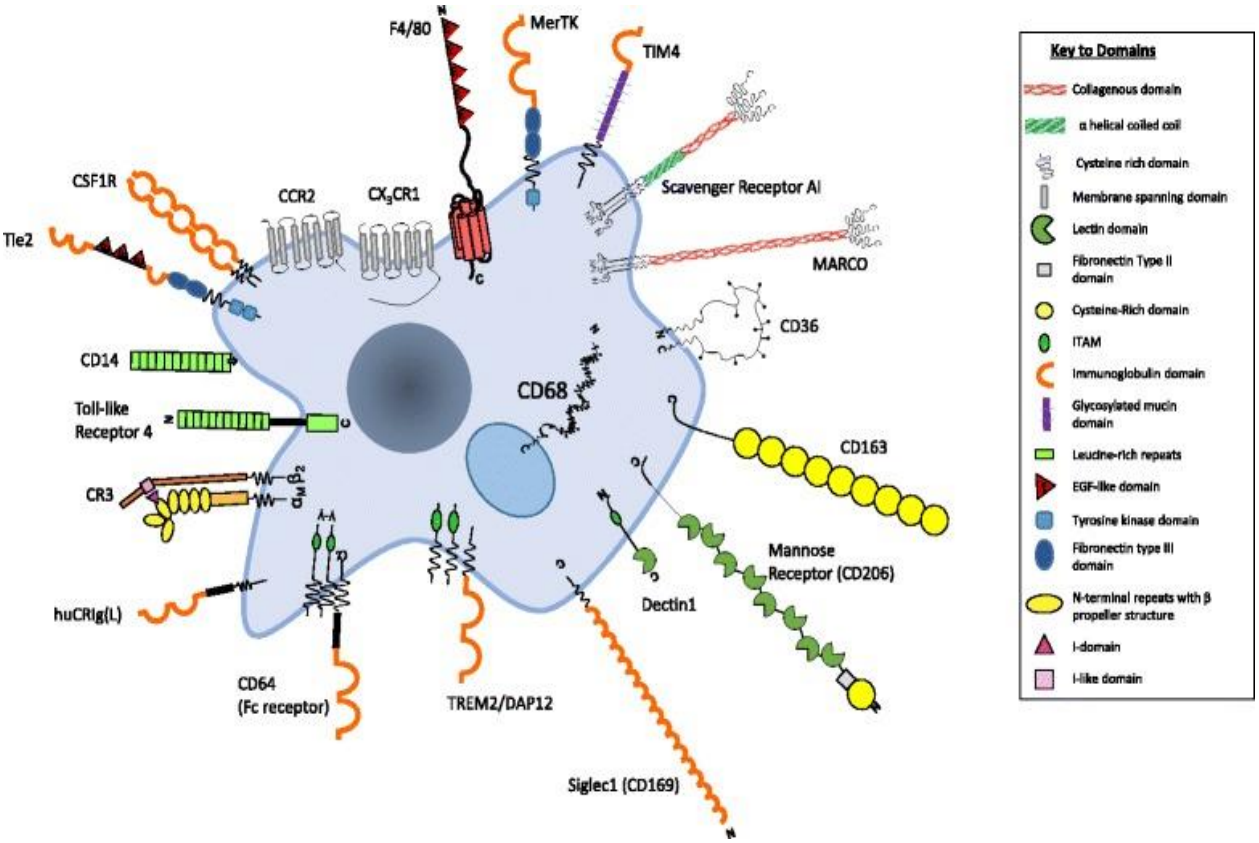

Figure 10. Tissue macrophage markers. Tissue macrophages express an array of markers depending on their niche and corresponding function[111].

of a spectrum, however, and what is key is macrophages' plasticity and ability to change their expression profile based on the needs at hand[109].

1.12 Mechanisms of macrophage infection and reservoir establishment

HIV entry into target cells requires binding to the CD4 receptor and either the CCR5 or CXCR4 co-receptors, as previously mentioned. Therefore, it is theoretically plausible that any cells expressing these markers could be infected by HIV. Macrophages fall under this classification, as they express both CD4, though at much lower levels than do T cells, in addition to CCR5[119]. Furthermore, the phagocytic role of macrophages in immunity means that they will engulf infected and dead CD4+ T cells which carry HIV. Both of these methods must be considered as possible routes of infection of macrophages during the HIV natural history. It has been shown that macrophages may contain the virus after phagocytosis of an infected T-cell[120]. The direct route of infection is a source of controversy in the field, however, in part due to intrinsic qualities of macrophages[121]. For instance, as stated above, macrophages express far less CD4 than T cells. In the case of untreated chronic infection, however, once the CD4+ T cell population is significantly depleted, macrophages can become targets of the virus[122]. Once HIV enters the macrophage, however, it is unclear if the virus is able to replicate and thus cause further infection, since macrophages express SAMHD1, a protein which degrades dNTPs and therefore inhibits the reverse transcriptase activity of HIV[84]. Interestingly, macrophages derived from female donors were less susceptible to *in vitro* HIV-1 infection than those derived from males due to their more robust expression of SAMHD1[46]. As mentioned above, efflux pumps such as p-glycoproteins and MRPs in macrophages may decrease the efficiency of ARVs in these cells, allowing for potential infection persistence during treatment[107,123], and macrophages are much less susceptible to apoptosis due to HIV infection[124].

1.13 Lungs as anatomical reservoirs of HIV

Prior to widespread implementation of ART, many AIDS- associated pathologies affected the lungs, such as pneumonia, alveolitis and tuberculosis (TB)[105,125]. Untreated HIV infection is characterized by significant inflammation and dysregulation of immune processes in the lung, as shown in Fig. 11. Indeed, the HIV-1-Ba-L strain commonly used in research was initially isolated from a lung tissue[126]. Following infection, an influx of immune cells occurs, including eosinophils and neutrophils, but also CD8+ T cells, which disrupts the CD4/CD8 ratio in this compartment

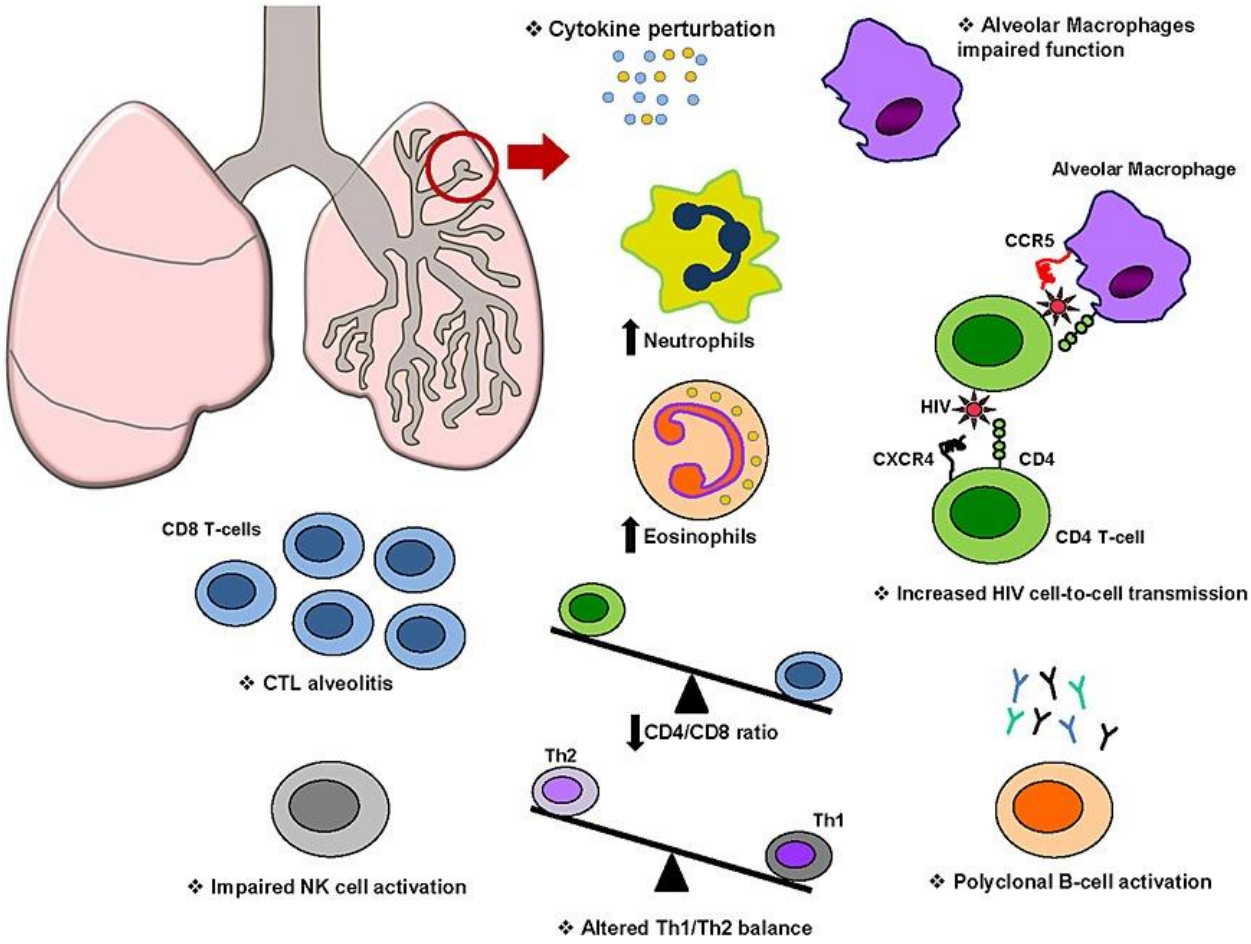

Figure 11. Effect of HIV infection on pulmonary immunity. Untreated HIV infection causes severe disruption to the immune environment of the lungs, including aberrant cytokine secretion, influx of CD8+ cytotoxic T cells and increased cell-cell HIV transmission[105].

and is the hallmark of the lymphocytic alveolitis described above[127]. This lymphocyte imbalance is further exacerbated by the characteristic depletion of CD4+ T cells, by apoptosis or killing by the incoming CTLs. Aberrant secretion of pro-inflammatory cytokines, such as IL-1β, IL-6, & TNF-α by AMs in the lung causes vast inflammation and stimulates B-cell activation and polyclonal expansion[105]. Due to the lungs' role in gas exchange, the tissue is made up of a vast network of narrow capillaries tightly packed with red blood cells to encourage slow cellular flow[128]. A consequence of this high-surface-area architecture is that it encourages cell-cell transmission of virus due to the prolonged contact.

In the current era of successful ART, we see the restoration of many of these lung immune environment disturbances; the CD4/CD8 T cell ratio is restored, immune activation is decreased, and opportunistic infections are less common[105]. Despite these improvements, however,

successfully ART-treated PLWH are far more susceptible to several pulmonary co-morbidities. The World Health Organization (WHO) reports up to a 27-fold increased risk for TB infection if a person is HIV+[129], though awareness of HIV status and access to ART varies by region. PLWH are also more likely to develop COPD[130]. So, while ART leads to a decreased HIV burden in the lungs there is some level of perturbation that makes PLWH susceptible to pulmonary disorders. Our group has shown that HIV DNA levels remain high in CD4+ T cells in the lung mucosa, on average 13 times higher than in CD4+ T cells found in the peripheral blood[104]. CD8+ T cells in the BAL are almost exclusively of an effector memory phenotype and are more activated and exhausted than peripheral CD8+ T cells[131]. Furthermore, our group also reported that double negative (DN) T cells, which do not express either CD4 or CD8, play a role in HIV reservoir seeding and maintenance in the lungs; DN T cells are more express more markers associated with HIV persistence and are less senescent in the BAL compared to the blood in PLWH[132]. All of these perturbations can contribute to the persistence of HIV in the pulmonary mucosa. As such, the lungs become a candidate for an anatomical reservoir of HIV warranting investigation. The importance of the interplay between immune regulators in the lungs is exemplified by the current COVID-19 pandemic. Indeed, it now seems that the most severe and fatal cases of COVID-19 are caused by the repercussions of cytokine storm, severe acute respiratory distress syndrome (ARDS), or exacerbation of an underlying medical condition, such as a cardiac disorder or diabetes[133]. Of course, lung immunity disruption is also closely linked to smoking, and it has been shown that in demographics such as those in Canada where HIV is more prevalent within a particular community, PLWH are more likely to be smokers[134,135].

1.14 Effect of smoking on lung immunity and inflammation

As previously mentioned, smoking is highly correlated with HIV+ status, and the chronic inflammation caused by regular smoking has a significant effect on the pulmonary immune environment, and AMs. Studies have shown that HIV+ status is a risk factor for acute exacerbation of COPD independent of smoking status[26], so it warrants discussing the specific effects attributed to smoking.

Smoking results in a significant increase in the AM population[136], whose metabolic and enzymatic processes are perturbed[110]and who show impaired phagocytic capabilities[137]. In parallel, an influx of CD8+ T cells causes a disruption of the CD4/CD8 ratio in the BAL of smokers, the same effect that is seen in acute HIV infection[138]. Furthermore, an increase in Th1 and Th17 cells, the majority

expressing the pro-inflammatory marker CCR6, can be seen in smokers' BAL[139]. While particular T cells are activated, B cell development is suppressed by cigarette smoke leading to a higher proportion of memory B cells in the lungs of smokers[140]. This significant immune disruption is reflected by the decrease of circulating IgA, IgG and IgM and increase in IgE which may contribute to the increased risk of allergy development among smokers. With the increase in availability of cannabinoids in many areas due to legalization, more data is needed on the effect of this substance on lung immunity; much less is known about the effects of cannabinoids *in vivo.* Since many people smoke cannabis recreationally, it can be difficult to parse effects of tobacco and cannabis. This is especially true in the context of HIV infection; as PLWH have high tobacco smoking rates[134,135] and often use medicinal cannabis[141]. As such, this project takes into consideration the smoking status of participants in evaluating the immunophenotype of AMs.

1.15 Alveolar macrophages as potential cellular reservoirs of HIV

AMs account for up to 95% of cells in the alveolar space in homeostasis and are the critical sentinels of the lung immune environment[128]. The lung mucosa is consistently exposed to antigens from the external environment and AMs are found on the epithelial surface, surveying and clearing particles, pathogens and other pollutants by phagocytosis and efferocytosis[108]. Phagocytosis involves engulfing the offending particle and breaking the contents down enzymatically, while efferocytosis refers to the degradation of apoptotic cells in such a way as to avoid inflammation. This is a critical role of the AM; particles must be cleared in a non-inflammatory manner to protect the integrity of gas exchange, which occurs in extremely fragile alveoli[117] (Fig. 12). Furthermore, in their role as immune cells they expand allergen-specific T-cell responses, and clear debris by removing senescent cells and helping to repair damaged tissue. Upon infection, monocytes are recruited from the periphery to differentiate into macrophages and assist with resolving the inflammation, so at any given time the pool of AMs in the lung mucosa will be a mixture of monocyte-derived and tissue-resident macrophages[142]. The complex factors contributing to tissue macrophage identity, including their ontogeny and function, was described above, and AMs are no exception. Some AMs have been shown to have a lifespan of several months and the capacity for self-renewal without infiltration of peripheral monocytes[113]. The longevity of AMs contributes gives us pause in the context of latent viral infection, as it is just one of the many characteristics that makes AMs a suitable candidate for a cellular reservoir of HIV. AMs express low levels of CD4 and CCR5, along with a host of other markers related to their role which are outlined below,

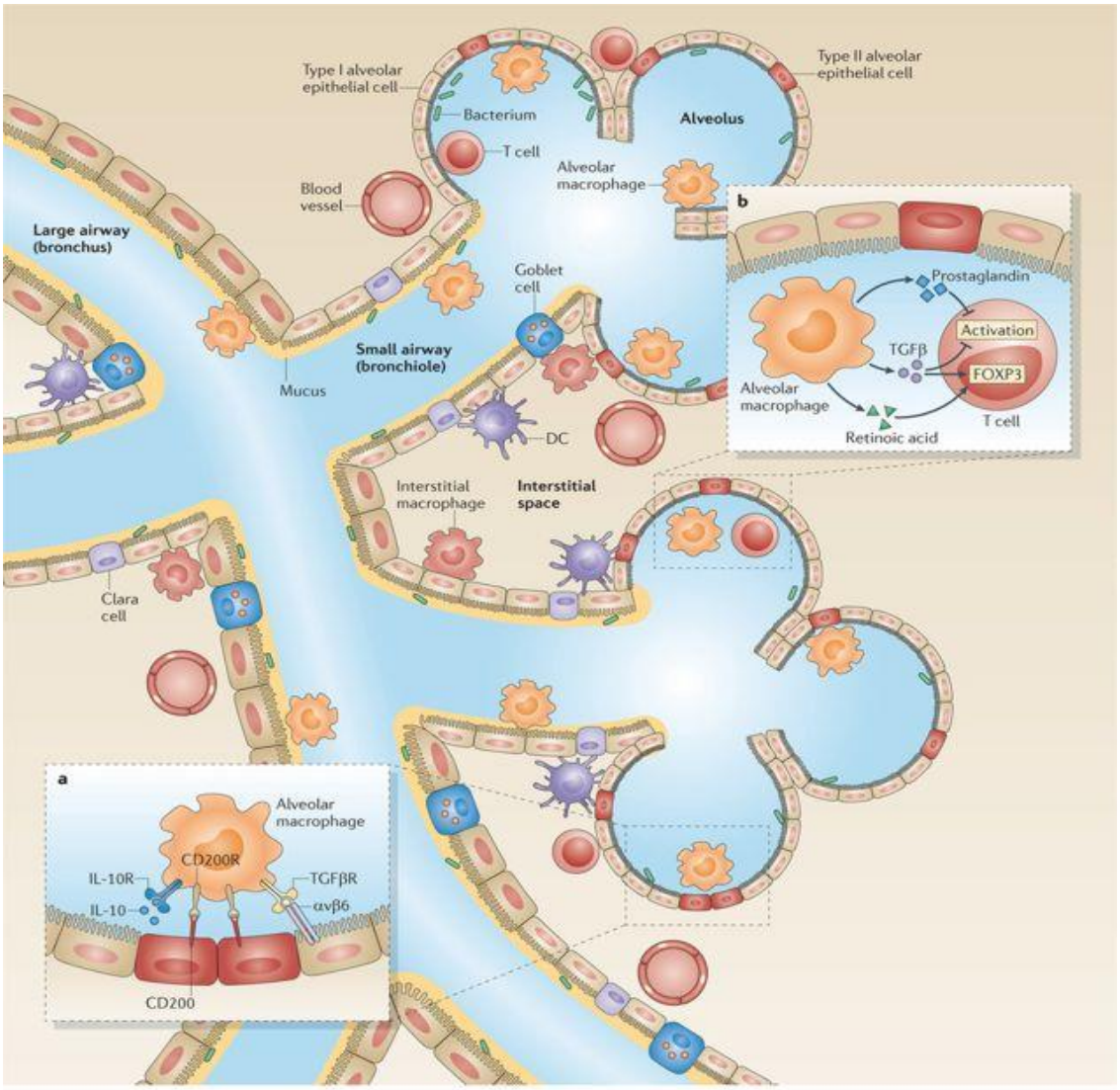

Figure 12. Role of alveolar macrophages in homeostasis. AMs act as sentinels in the alveolar space, patrolling and clearing debris and pathogens by efferocytosis without compromising the integrity of gas exchange[117].

so theoretically HIV should be able to bind and enter these cells if it comes into contact. Phagocytosis is another potential route of infection, as AMs will engulf infected CD4+ T cells. This latter mechanism has been demonstrated as a route of macrophage infection with HIV *in vitro* and with SIV[120] (Fig. 13). The question of whether AMs can be directly infected by HIV or solely via phagocytosis of infected CD4+ T cells has not been addressed and is one of the aims of this project. Notably, macrophages are more resistant to HIV-induced cell death, partly due to expression of factors such as SAMHD1[124]. This host restriction factor sequesters dNTPs, inhibiting reverse transcriptase activity of HIV, so that the virus may be integrated but not efficiently

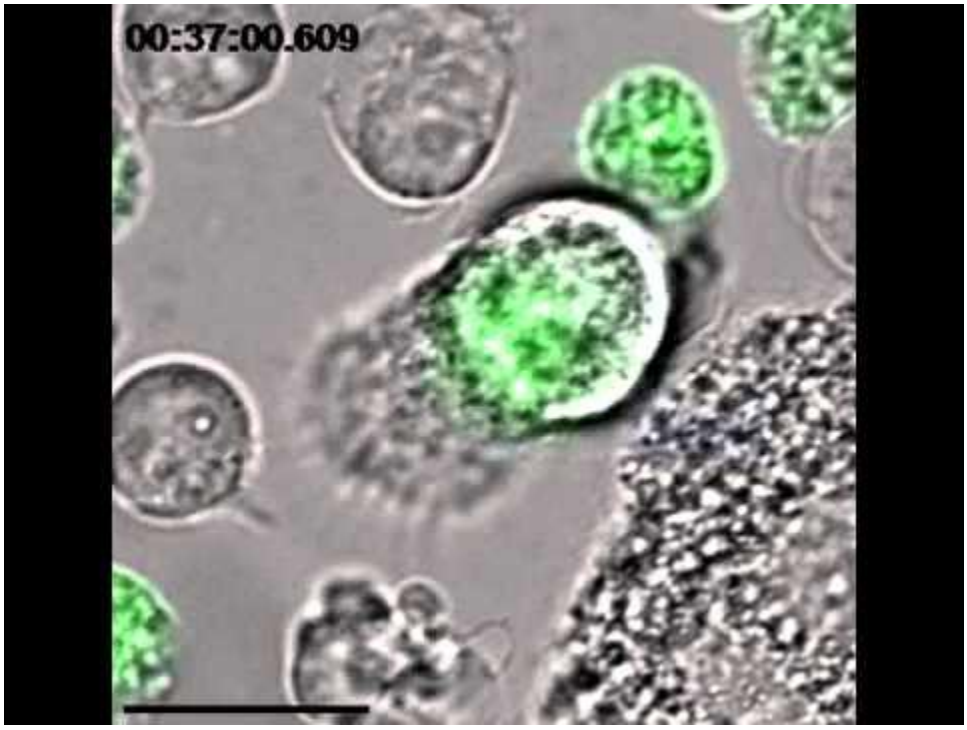

Figure 13. Macrophage engulfment of HIV-infected CD4+ T cell. One suggested route of HIV infection of macrophages is via phagocytosis of infected CD4+ T cells, as shown in this still image from a video showing an MDM engulfing an HIV-infected Jurkat cell (green)[120].

replicated. Furthermore, efflux pumps may restrict penetration of some ARVs, namely NRTIs and PIs, into these cells, ameliorating viral persistence under treatment[106].

When studying the HIV reservoir burden in the lungs, our group recently showed that in ART-treated infection CD4+ T cells from BAL contained higher levels of HIV DNA than did CD4+ T cells from matched peripheral blood. However, when the same analysis was performed in AMs the results were more ambiguous as DNA levels varied greatly depending on the donor[104]. The AMs, therefore, require further study in the context of the latent HIV reservoir.

AMs express a host of proteins to perform their functions; the CD206 mannose receptor is a canonical marker of AMs. This PRR can recognize the mannan-coated cell walls of certain microorganisms, an example of a pathogen-associated molecular pattern (PAMP)[143]. AMs also express other 'scavenger receptors' such as the macrophage receptor with collagenous structure MARCO; CD169/ Siglec-1, a sialic acid receptor; and CD163, which binds haemoglobin[144-146]. A recent study in BAL fluid from ART-naïve HIV-infected individuals showed that the frequency of CD163+ AMs was reduced compared to HIV- controls, which correlated with a shift toward a pro-inflammatory state and increased HLA-DR expression[147]. In addition to these, they also express

markers associated with their role as antigen presenting cells, such as HLA-DR[110]. As with all tissue-resident macrophages, this particular expression profile is highly nuanced and reflects the very particular functions of AMs. It follows, therefore, that this profile may change when the demands on the cell population change, such as in the context of viral infection. The converse also stands to reason, that if particular phenotypic signatures can be identified as unique to AMs from PLWH, this could give some insight into what the pulmonary immune environment looks like, specifically now in the post-ART era. The current project seeks to address this question by performing a comprehensive phenotypic analysis of AMs from both HIV- and HIV+ individuals, specifically those without pulmonary symptoms to understand what underlying phenotype may be present solely due to HIV seropositive states.

Chapter 2: Methods

2.1 Study Population

Donors were recruited from the Chronic Viral Illness Service at the Glen site of the McGill University Health Centre (MUHC) as well as the MUHC Respirology clinic into one of 4 cohorts, with n=7 per group:

- HIV- non-smokers
- HIV- smokers
- HIV+ non-smokers
- HIV+ smokers

All participants had to be free of pulmonary symptoms, chronic lung disease (such as asthma or chronic obstructive pulmonary disease) and HIV+ individuals were required to be successfully treated with antiretroviral therapy. HIV+ participants were required to have suppressed viral load for ≥ 3 years with a CD4 count ≥ 350 cells/mm^3.

2.2 Ethics Statement

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

2.3 Bronchoalveolar Lavage

Participants underwent bronchoscopy, performed by a licensed respirologist, to obtain bronchoalveolar lavage (BAL) fluid[148] (Fig. 14). Participants' heart rate and rhythm were monitored using cardiac leads applied to the chest and oxygen saturation was monitored using an

Figure 14. Bronchoscopy. Schematic of positioning of bronchoscope used before flushing of alveolar space during bronchoalveolar lavage (BAL)[148].

oxygen probe applied to the first finger of a hand. Participants were first administered topical anaesthetic to the back of the throat, then given midazolam 0.01 – 0.04 mg/kg and fentanyl 50 – 100 mcg sedation intravenously in the presence of a respirologist or anesthetist. Bronchoscopy was performed using a flexible bronchoscope and advancing until positioned in a desired sub-segmental bronchus.

Lavage was then performed by the respirologist by instilling saline, approximately 50mL at a time by syringe, then applying gentle suction. The lavage fluid was collected in the syringe and then transferred to a collecting container. This was repeated to collect approximately 100mL of BAL fluid and the fluid was kept on ice until processed.

2.4 Isolation of pulmonary mucosal cells from BAL fluid

BAL fluid was processed as described in our recent method paper, Salahuddin, Thomson *et al.*[149], on which I am co-first author. Briefly, BAL fluid was vortexed and centrifuged at 200 *x g* and the supernatant aliquoted and stored at -80°C for potential use in other downstream assays. The remaining BAL cells were then resuspended in RPMI 1640 (Wisent Bioproducts, Saint-Jean-Baptis te, QC, Cat# 350-000-CL) containing 10% fetal bovine serum (FBS) (Wisent Bioproducts, Saint-Jean-Baptiste, QC Cat# 080-150) and 1% penicillin/streptomycin (Wisent Bioproducts, Saint-Jean-Baptiste, QC, Cat# 450-201-EL) for downstream use (Fig. 15).

2.5 Isolation of PBMCs from whole blood

Whole blood from participants was processed as described in Salahuddin, Thomson *et al.*[149], of which I am co-first author. Briefly, blood was centrifuged at 300 *x g* for 15 minutes to separate plasma, which was then removed, aliquoted and stored at -80°C for potential use in other

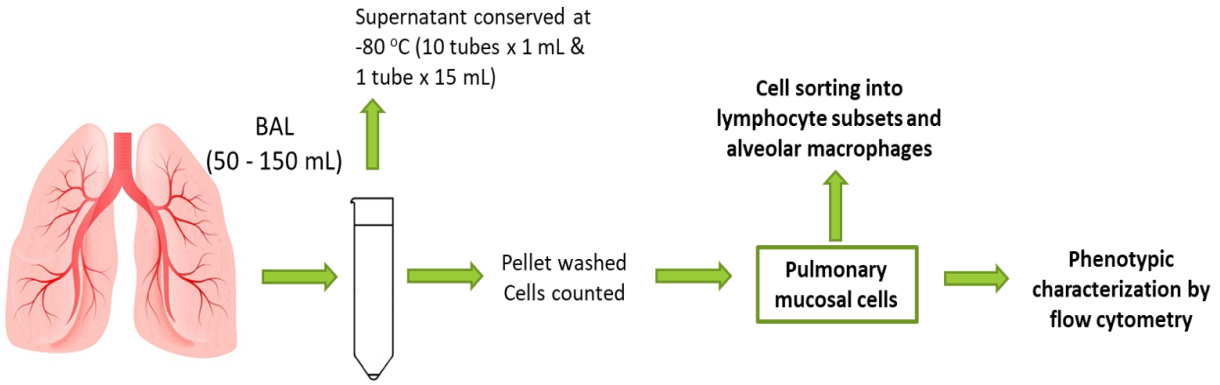

Figure 15: Isolation of pulmonary mucosal cells from bronchoalveolar lavage fluid. 'BAL' denotes bronchoalveolar lavage fluid[149].

downstream assays. The remaining blood was then diluted with RPMI 1640 and layered over Lymphocyte Separation Medium (Wisent Bioproducts, Saint-Jean-Baptiste, QC, Cat# 305-010-CL) and centrifuged at 600 *x g* for 25 minutes with no brake. The PBMCs were then extracted from the interface of the two liquid phases and washed with RPMI 1640 before resuspension in RPMI 1640 containing 10% FBS and 1% penicillin/streptomycin for downstream use (Fig. 16).

2.6 Immunophenotyping of AMs and peripheral blood monocytes

To assess AM and peripheral blood monocyte morphology, cells were stained with the antibodies and panels outlined in Tables 1-3. AM immunophenotyping markers were divided into two panels to circumvent issues with autofluroescence by avoiding use a full 14-colour panel while a single panel could be used monocyte immunophenotyping as PBMCs do not pose a problem in terms of their autofluorescence and fewer markers were assessed. Stained cells were acquired using the BD Fortessa X20 and results analyzed using FlowJo version 10 (FlowJo LLC, Ashland, OR).

A t-distributed stochastic neighbor embedding (t-SNE) calculation was performed on a sub-population of cells from two donors. Cells of interest within whole BAL cells were identified by gating single, live, CD45+ cells and performing the t-SNE calculation on this population. This organized all remaining parameters into two parameters (tsne-x and tsne-y) and clusters the cells according to similarity of expression profile. Within the tsne-x vs. tsne-y plot a third parameter can then be applied as a heat map to observe the strength of expression of that particular marker among the various clusters of cells.

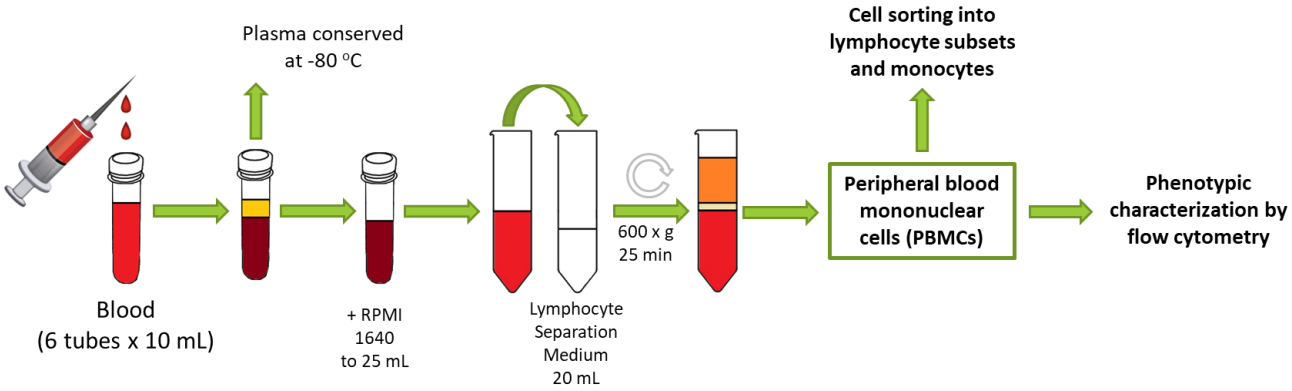

Figure 16: Isolation of peripheral blood mononuclear cells (PBMCs) from peripheral blood[149].

Table 1. Immunophenotyping markers used for characterization of AMs from whole BAL cells – panel 1

Marker	Fluorochrome	Clone	Company	Description
Live/Dead Fixable Aqua Stain	405nm excitation	N/A	Invitrogen, Life Technologies Corporation, Eugene, OR	Viability stain which can enter dead or dying cells whose membrane is permeabilized
CD45	PE-Cy5	HI30	BD Pharmingen, BD Biosciences, San Jose, CA	Receptor protein tyrosine phosphatase found on all cells of haematopoietic origin
HLA-DR	BV605	G46-6	BD Horizon, BD Biosciences, San Jose, CA	MHC class II molecule used for antigen presentation
CD206	PE	19.2	BD Pharmingen, BD Biosciences, San Jose, CA	Mannose receptor highly expressed on AMs
CD169	BB515	7-239	BD Horizon, BD Biosciences, San Jose, CA	Sialoadhesin expressed on alveolar but not interstitial macrophages
CD163	APC/Cyanine7	GHI/61	BioLegend, San Diego, CA	Scavenger receptor which binds hemoglobin complexes
CD14	BV786	M5E2	BD Horizon, BD Biosciences, San Jose, CA	LPS receptor highly expressed on classical monocytes
CD16	Alexa Fluor 700	3G8	BD Pharmingen, BD Biosciences, San Jose, CA	Fcγ receptor expressed on non-classical monocytes

CD33	BV650	WM53	BD OptiBuild, BD Biosciences, San Jose, CA	Sialoahdesin expressed on peripheral myeloid cells
CCR2 (CD192)	PE/Dazzle 594	K036C2	BioLegend, San Diego, CA	Chemokine receptor associated with classical monocyte recruitment

Table 2. Immunophenotyping markers used for characterization of AMs from whole BAL cells – panel 2

Marker	Fluorochrome	Clone	Company	Description
Live/Dead Fixable Aqua Stain	405nm excitation	N/A	See Table 1	See Table 1
HLA-DR	PE/Dazzle 594	G46-6	BioLegend, San Diego, CA	MHC class II molecule used for antigen presentation
CD206	PE	19.2	See Table 1	See Table 1
CD169	BB515	7-239	See Table 1	See Table 1
CD163	APC/Cyanine7	GHI/61	See Table 1	See Table 1
CX3CR1	BV421	2A9-1 (Rat)	BD Horizon, BD Biosciences, San Jose, CA	Chemokine receptor associated with non-classical monocyte recruitment
CD71	APC	M-A712	BD Pharmingen, BD Biosciences, San Jose, CA	Transferrin receptor expressed on all macrophages
IL-4Rα (CD124)	PE/Cyanine7	G077F6	BioLegend, San Diego, CA	Receptor for IL-4 cytokine expressed on some tissue macrophage HIV reservoir cells

Ki-67	BV711	B56	BD Horizon, BD Biosciences, San Jose, CA	Nuclear protein expressed during mitosis to indicate proliferating cells
c-Kit (CD117)	PerCP/Cyanine5.5	104D2	BioLegend, San Diego, CA	Mast/stem cell growth factor
CCR5 (CD195)	BV605	2D7/CCR5	BD Horizon, BD Biosciences, San Jose, CA	Chemokine receptor which acts as co-receptor for HIV

Table 3. Immunophenotyping markers used for characterization of monocytes from PBMCs

Marker	Fluorochrome	Clone	Company	Description
Live/Dead Fixable Aqua Stain	405nm excitation	N/A	See Table 1	See Table 1
HLA-DR	BV605	G46-6	See Table 1	See Table 1
CD3/19/20/56	APC	UCHT1, HIB19, 2H7, 5.1H11	BD Pharmingen, BD Biosciences, San Jose, CA	Lineage marker to exclude T cells (CD3), B cells (CD19, CD20) and NK cells (CD56)
CD33	BV650	WM53	See Table 1	See Table 1
CD14	BV786	M5E2	See Table 1	See Table 1
CD16	Alexa Fluor 700	3G8	See Table 1	See Table 1
CD169	BB515	7-239	See Table 1	See Table 1
CD163	APC/Cyanine7	GHI/61	See Table 1	See Table 1
CCR2 (CD192)	PE/Dazzle 594	K036C2	See Table 1	See Table 1
CX3CR1	BV421	2A9-1 (Rat)	See Table 2	See Table 2
Ki-67	BV711	B56	See Table 2	See Table 2

IL-4Rα (CD124)	PE/Cyanine7	G077F6	See Table 2	See Table 2
c-Kit (CD117)	PerCP/Cyanine5.5	104D2	See Table 2	See Table 2

2.7 Statistical analyses

For immunophenotyping data, Mann-Whitney unpaired non-parametric statistical testing was applied to HIV+ versus HIV- specimens for each pair of markers examined (ex. CD14-CD16-) to identify if differences between frequencies were statistically significant. GraphPad Prism version 8.01 software (GraphPad Software, La Jolla, CA) was used for all statistical analyses and to generate all graphs of immunophenotyping results.

2.8 Differentiation of MDMs

Monocytes from PBMCs were differentiated into MDMs *in vitro* for use in infection optimization protocols. PBMCs were isolated from fresh blood of a healthy donor as described above and cells were plated either in a 10 cm untreated petri dish at ~25 million PBMCs/plate or in a 24-well plate at ~ 1 million PBMCs/well in RPMI 1640 containing 1% penicillin/streptomycin but no serum. Cells were allowed to adhere for 3h. The non-adherent cells were then removed (these peripheral blood lymphocytes could be used for other assays if needed or discarded), the remaining adherent cells washed gently once with RPMI 1640 containing 1% penicillin/streptomycin and then RPMI 1640 containing 10% Human AB serum (Wisent Bioproducts, Saint-Jean-Baptiste, QC, Cat# 022-210), 1% penicillin/streptomycin and 25ng/mL M-CSF (GenScript, Piscataway, NJ, Cat# Z02924) was added; 10mL/plate for 10cm plate or 1mL/well for 24-well plate. Half the media was replaced with RPMI 1640 containing 10% Human AB serum and 1% penicillin/streptomycin after 3-4 days and the cells were differentiated at Day 6-7. Cells were maintained in culture with RPMI 1640 containing 10% FBS and 1% penicillin/streptomycin, replenishing the media every 3-4 days. MDMs were detached from plates by removing media, washing cells 3X with phosphate-buffered saline (PBS) (Wisent Bioproducts, Saint-Jean-Baptiste, QC, Cat# 311-010-CL) and then adding CellStripper (Corning, Wiesbaden, Germany, Cat# 25056CI); 3mL for a 10cm plate or 300µL for a well in a 24-well plate. MDMs were incubated with the CellStripper for 2h at 37°C, 5% CO_2 and then detached by gentle scraping with a P1000 pipette tip.

2.9 Characterization of 'Daisy' AM-like macrophage cell line

Due to the COVID-19 pandemic and interruption of participant recruitment, a recently characterized AM-like cell line was substituted for fresh primary AMs isolated from BAL. These 'Daisy' cells are a sub-clone of THP-1 cells which are macrophage pre-cursor cells in suspension that require mitogenic stimulation to become adherent and macrophage-like. Daisy cells, however, predominantly adhere in a monolayer when cultured, without mitogenic stimulation, and were found to express high levels of CD206 by Sadofsky and colleagues[150]. An aliquot of this cell line was kindly gifted by Dr. Laura Sadofsky (University of Hull, UK) for our use. THP-1 cells were also used to perform a similar phenotypic comparison as conducted in Sadofsky *et al*.[150]. THP-1 and Daisy cells were cultured in RPMI 1640 containing 10% FBS and 1% penicillin/streptomycin. For stimulation of THP-1 cells, 50nmol/L of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, MO, Cat# P1585) was added to the media of THP-1 cells plated at 330,000 cells/mL in a 6-well plate. Cells were stimulated for 24h, then media was aspirated and cells were washed 3X with PBS before fresh media was added. Cells were rested for 24h and then harvested for staining for flow cytometry. Sadofsky *et al.* evaluated the expression of various known macrophage markers, such as CD11b, CD11c, CD14, CD16, CD64, CD80, CD86, CD163, CD169, CD206 and CX3CR1. We characterized the expression of some of these markers, in addition to others relevant to HIV, on Daisy cells, THP-1 cells and stimulated THP-1 cells. CD206, HLA-DR, CD163, CD169, CD14, CD16, CD33, CCR2, CX3CR1, CCR5, CD4 and CD11b markers were used as described above, in addition to CXCR4-PE-Cyanine7 (Clone 12G5, BD Pharmingen, BD Biosciences, San Jose, CA). Cells were stained using the same protocol as described above for immunophenotyping.

2.10 *In vitro* infection of MDMs and Daisy cells with HIV strains

I optimized an *in vitro* infection protocol using MDMs and the HIV strain NL4.3-Bal-IRES-HSA, kindly gifted from Dr. Michel Tremblay at Université Laval. This R5-tropic NL4.3-Bal-IRES-HSA laboratory strain of HIV with an integrated reporter gene murine heat-stable antigen (HSA) allows for infection readout by flow cytometry as HSA remains on the surface of infected cells and is detectable by CD24 antibody[151]. Daisy cells[150] were used as a substitute for primary AMs after COVID-19 pandemic prevented participants coming to hospital for BAL.

The finalized protocol involved plating MDMs or Daisy cells in a 24-well plate at ~100,000-200,000 cells/well. Media was removed from the wells and replaced with RPMI 1640 containing

10% FBS and 1% penicillin/streptomycin, and 50ng of p24 per million cells from a particular viral stock. Three HIV strains were used; an R5-tropic NL4.3-Bal-IRES-HSA, an X4-tropic NL4.3, and a strain pseudotyped with the envelope of vesicular stomatitis virus (VSV), NL4.3-VSV. The NL4.3 strain was a kind gift from Dr. Petronela Ancuta at the Centre de recherche du CHUM and the NL4.3-VSV strain was a kind gift from Dr Benoit Barbeau at the Université du Québec à Montréal. This latter HIV strain bypasses the need for CD4 and co-receptor binding for entry, as the VSV allows the strain to be internalized by clathrin-mediated endocytosis[152]. Cells were incubated with virus for 3h at 37°C, 5% CO_2, then media was removed and cells gently washed 2X with RPMI 1640 containing 10% FBS and 1% penicillin/streptomycin and then 1mL of this same media was added to the well. Readout of infection was done by flow cytometric evaluation of intracellular p24 expression usually on day 2/3 for NL4.3-VSV and on days 3, 7 and 10 of infection for other strains. The viral strain pseudotyped with VSV performs only a single round of replication, and therefore infection is detected most robustly within the first 72h. Intracellular p24 and extracellular CD24 expression was evaluated for all strains, though CD24 expression should only be seen with NL4.3-Bal-IRES-HSA infection, as this was the only strain containing HSA, detectable by CD24.

Use of the NL4.3-Bal-IRES-HSA allows for extracellular probing of the heat stable antigen (HSA) or CD24, expressed on infected cells. Following detachment as described above, MDMs and Daisy cells were stained with Live/Dead Fixable Aqua Stain, CDllb-APC (Clone ICRF44, BD Pharmingen, BD Biosciences, San Jose, CA), CD4-BV650 (Clone L200, BD Horizon, BD Biosciences, San Jose, CA) and CD24-PE (Clone M1/69 (rat), BD Pharmingen, BD Biosciences, San Jose, CA). Following the extracellular stain, cells were permeabilized using BD Cytofix/Cytoperm (BD Biosciences, San Jose, CA, Cat# 554714) and intracellular staining with HIV-1 Core Antigen-FITC (p24; Clone KC57, Beckman Coulter, Brea, CA) was performed. Stained cells were then acquired using the BD Fortessa X20 and results analyzed using FlowJo version 10.

Chapter 3: Results

3.1 Study population

Participants were recruited for the bronchoscopy and blood collection through the Chronic Viral Illness Service (CVIS) at the Glen site of the McGill University Health Centre (MUHC). Participant characteristics are summarized in Table 4. Among HIV+ participants, the median age was 58 (Interquartile range (IQR) 3) years, the median duration of HIV infection was 9 (14) years, the median number of years on antiretroviral therapy (ART) with undetectable viral load was 7 (7) and the median CD4 count at the time of bronchoscopy was 633 (132) cells/mm^3. In the case of HIV- participants the median age was 29 (11) years, and the median CD4 count at the time of bronchoscopy was 761.5 (491.3) cells/mm^3. Participants recruited were generally from the men who have sex with men (MSM) community in Montreal and there was a requirement to have a suppressed viral load for a minimum of 3 years. The HIV- individuals were often acquaintances of individuals who have participated in clinical research at the clinic. Due to the COVID-19 pandemic which began in the midst of this project, participant recruitment was interrupted in January 2020 and at the time of writing, in December 2020, has not resumed.

Table 4. Summary of characteristics of participants who donated BAL and blood▲

Factor	HIV+ ART-treated individuals n = 9	HIV- individuals n = 7
Age in years, median (IQR)	58 (3)	29 (11)
Sex		
Male, n (%)	9 (100)	7 (100)
Ethnicity		
Caucasian, n (%)	7 (78)	7 (100)
Black – Caribbean, n (%)	2 (22)	0 (0)
Duration of HIV in years, median (IQR)	9 (14)	N/A
Nadir CD4 Count in count/mm^3, median (IQR)	216 (246.5)	N/A
CD4 Count at bronchoscopy in count/mm^3, median (IQR)	633 (132)	761.5 (491.3)
ART regimen		
NRTIs or NNRTIs, n (%)	9 (100)	N/A

Integrase inhibitors, n (%) Protease inhibitors, n (%)	8 (89) 2 (22)	N/A N/A
Duration of undetectable viral load in years, median (IQR)	7 (7)	N/A
Tobacco use, n (%)	4 (44)	2 (29)
Cannabis use, n (%)	3 (33)	1 (14)

▲ 'n' denotes number of participants in cohort with this characteristic
'IQR' denotes interquartile range

3.2 Morphology of cells recovered from bronchoalveolar lavage (BAL)

Cells collected from BAL comprise immune cells such as macrophages and lymphocytes, as well as red blood cells as shown in Fig. 17.

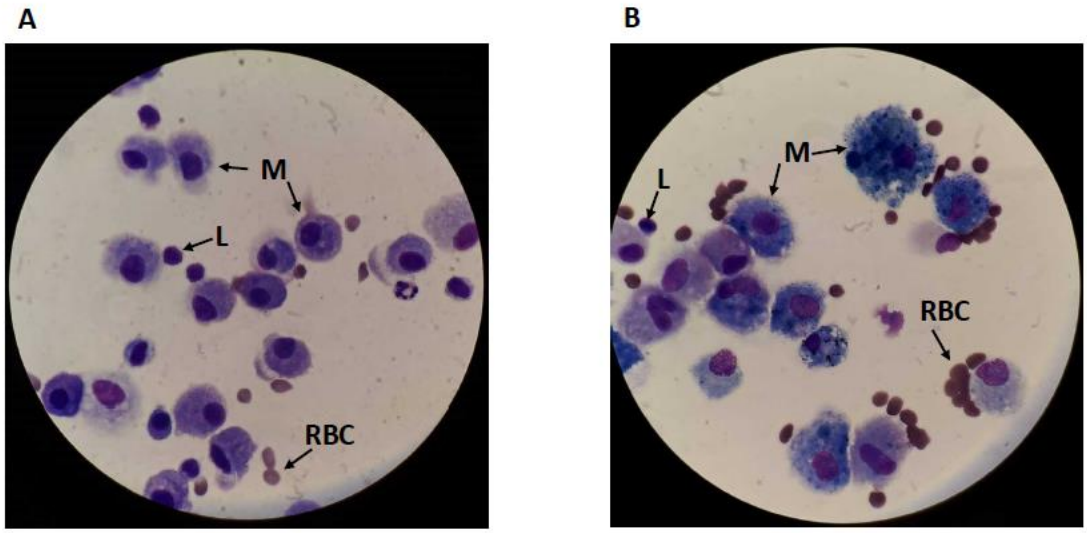

Figure 17. Microscope image of cells recovered from BAL. Spirometry images showing whole BAL cells from A. a non-smoker and B. a smoker, both HIV+. Cell types are indicated with arrows; 'M' denotes macrophages, 'L' denotes lymphocytes and 'RBC' denotes red blood cells.

3.3 Immunophenotyping

All subsequent analyses were done within AMs or non-AM myeloid cells as defined by the gating strategy shown in Fig. 18. In parallel, myeloid cells in PBMCs were also analyzed. Monocytes were identified as shown in the gating strategy in Fig. 19, as was as a particular population of myeloid cells with dimmer expression of the sialoadhesin CD33. A t-distributed stochastic

neighbor embedding (t-SNE) calculation was performed to identify clusters of cells with similar expression patterns among a pool of AMs and demonstrate their heterogeneity between donors (Fig. 20).

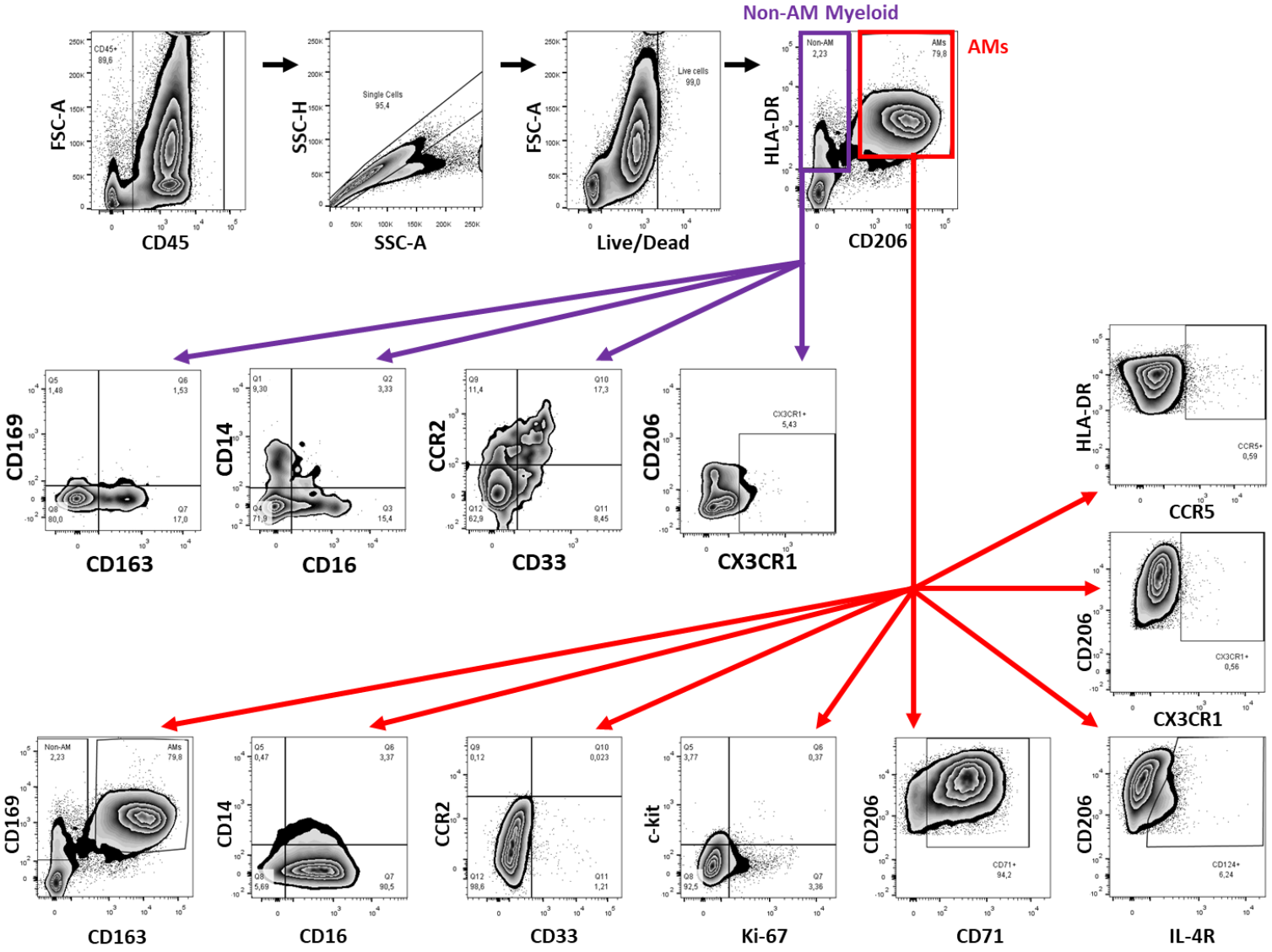

Figure 18. Flow cytometric gating strategy for alveolar macrophages (AMs) and non-AM myeloid cells. AMs defined as live, single, CD45+HLA-DR+CD206+ cells. Within this population, frequencies of CD169, CD163, CD14, CD16, CCR2, CD33, c-Kit, Ki-67, CX3CR1, CD71, IL-4R and CCR5 markers were quantified. Non-AM myeloid cells defined as live, single, CD45+HLA-DR+CD206- cells. Within this population, frequencies of CD169, CD163, CD14, CD16, CCR2, CD33, and CX3CR1 markers were quantified.

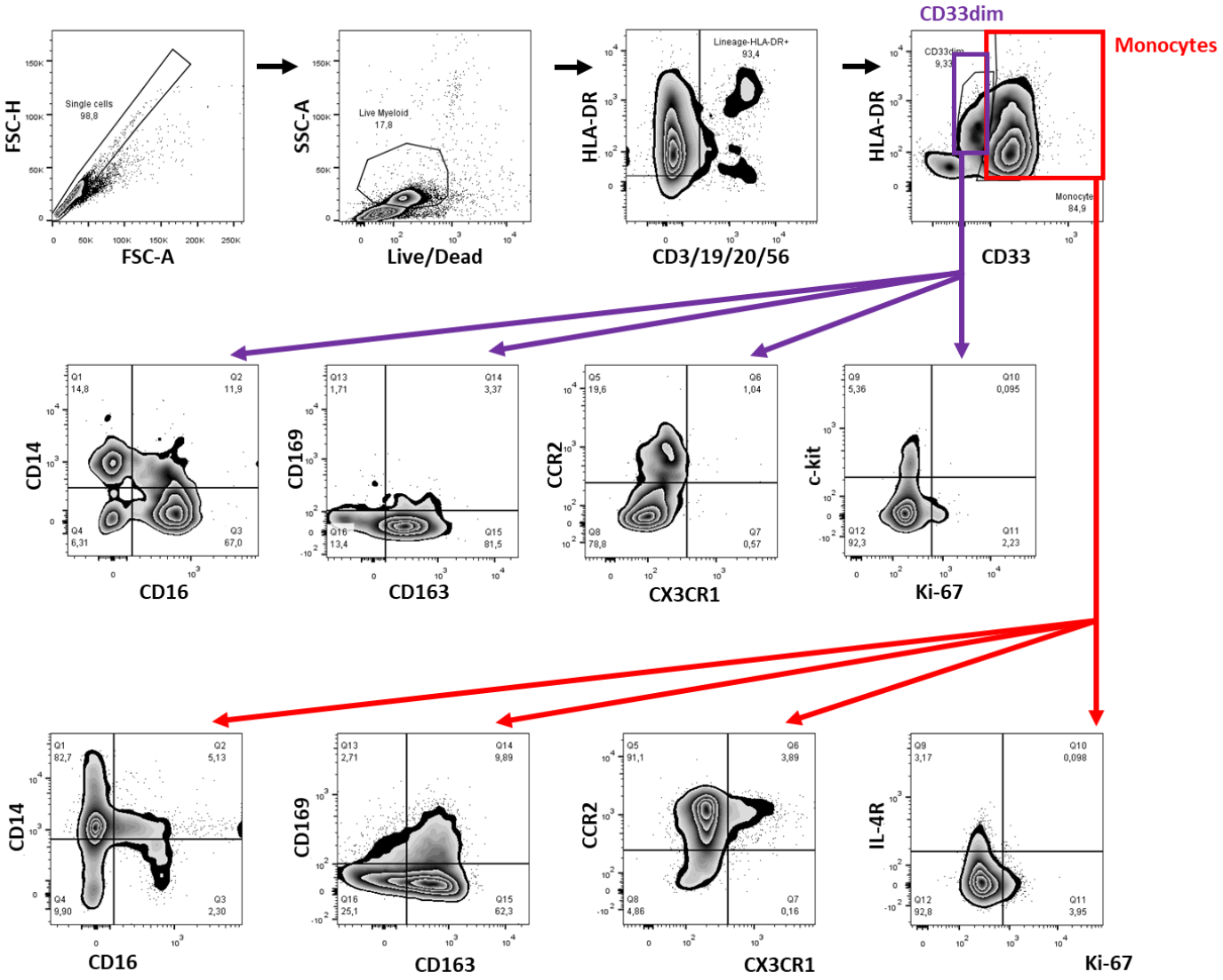

Figure 19. Flow cytometric gating strategy for monocytes and CD33dim myeloid cells within PBMCs. Monocytes defined as live, single, HLA-DR+Lineage-CD33+ myeloid cells. Within this population frequencies of CD14, CD16, CD169, CD163, CCR2, CX3CR1, IL-4R and Ki-67 markers were quantified. CD33dim myeloid cells defined as live, single, HLA-DR+Lineage-CD33dim myeloid cells. Within this population frequencies of CD14, CD16, CD169, CD163, CCR2, CX3CR1, c-Kit and Ki-67 markers were quantified. Lineage includes CD3, CD19, CD20 and CD56 markers.

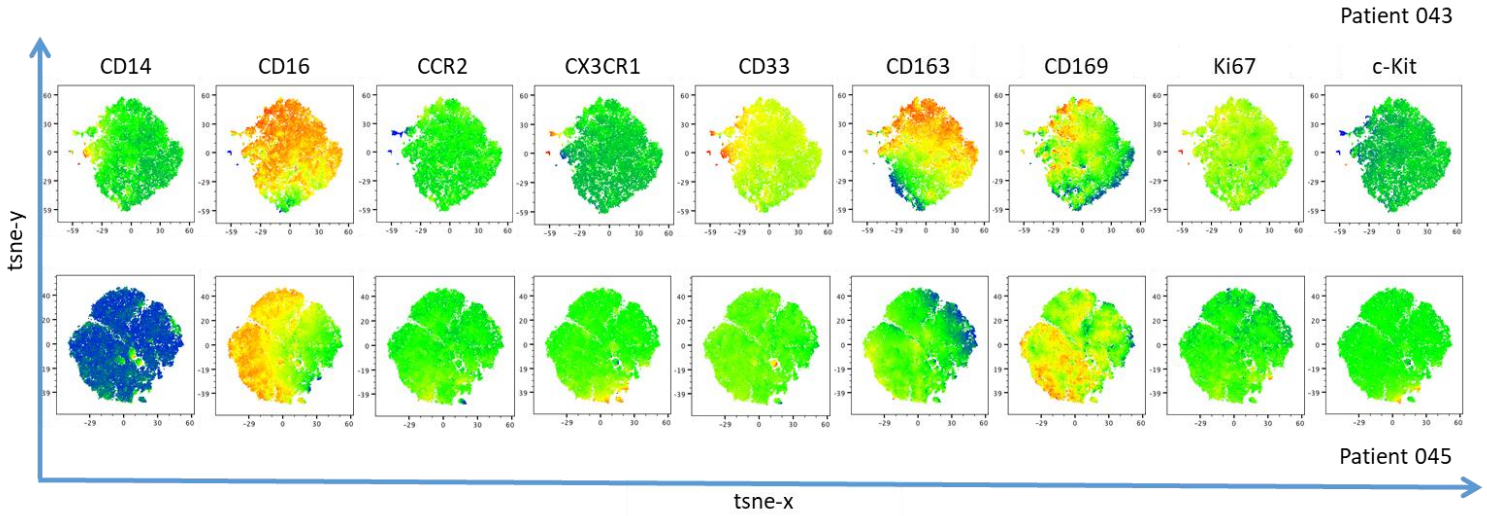

Figure 20. t-SNE plot of whole BAL cells. t-distributed stochastic neighbor embedding plot of sub-population of whole BAL cells gated on single, live, CD45+ cells with heat map of additional markers showing expression distribution for two HIV+ non-smokers.

3.3.1 Comparison of frequency of marker expression between HIV$^+$ and HIV- participants

Within the AM or monocyte populations, identified as explained above, the frequencies of the various markers of interest were analyzed. Markers were paired based on their relevance and the frequency of cells expressing one or both of these markers among the parent population (AMs or monocytes, for example) was identified. In the following figures, red dots are used to indicate those samples which came from smokers. Firstly, the expression of CD14 and CD16 expression was examined. The highest frequency of AMs exhibited a CD14-CD16$^+$ expression profile in both HIV+ and HIV- individuals (HIV+ mean±SD: 83.8±8.2%; HIV-: 86.8±11.8%) (Fig. 21A), and there was a trend within HIV+ individuals for smokers to have a higher frequency of CD14-CD16+ AMs (Fig. 21A). This CD14-CD16+ profile was less frequent in Non-AM myeloid cells (HIV+: 31.84±27.0%; HIV-: 28.3±27.5%), and the highest frequency of cells exhibited a CD14-CD16- profile in both HIV+ and HIV- individuals (HIV+: 48.4±18.4%; HIV-: 64.6±28.0%) (Fig. 21B). Meanwhile, within PBMCs, the monocyte population showed the expected expression pattern commonly seen in peripheral blood monocytes in both HIV+ and HIV- participants[153]; predominantly CD14+CD16- cells (HIV+: 72.8±3.4%; HIV-: 79.9±6.0%; p = 0.065) (Fig. 21C). Meanwhile, within the CD33dim population, which were those myeloid cells, still HLA-DR+, which showed less bright expression of CD33, the CD16+ populations dominated, with the CD14-

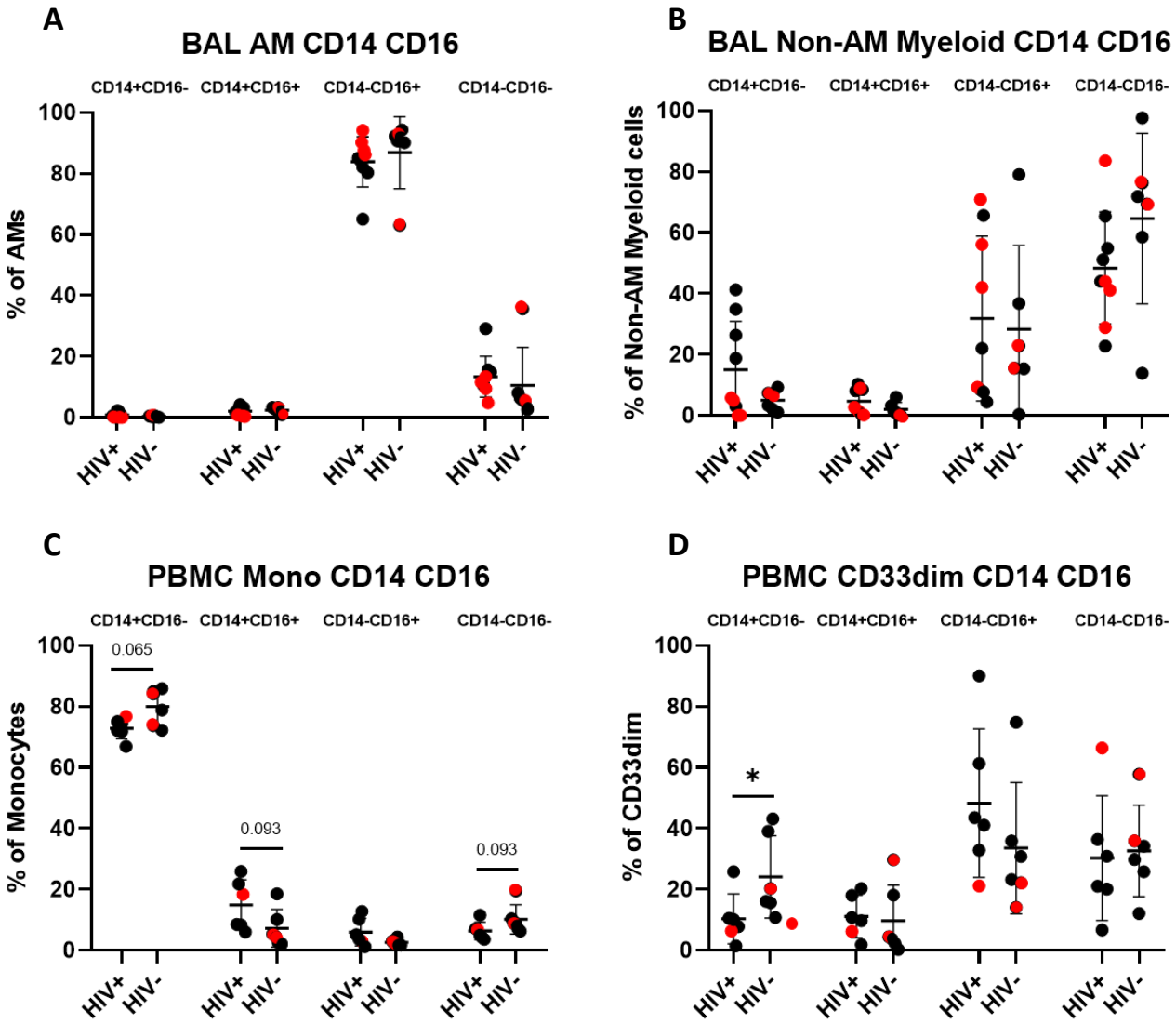

Figure 21. Classic myeloid marker expression in BAL and PBMC subsets. Frequency of CD14 and CD16 expression in A. AMs and B. Non-AM myeloid cells from BAL compared to C. monocytes and D. CD33dim myeloid cells from PBMCs. AMs defined within whole BAL cells as single, live, CD45+HLA-DR+CD206+ cells. Non-AM myeloid cells defined within whole BAL cells as single, live, CD45+HLA-DR+CD206- cells. Monocytes defined within PBMCs as single, live, myeloid, CD3-CD19-CD56-HLA-DR+CD33+ cells. CD33dim myeloid cells defined within PBMCs as single, live, myeloid, CD3-CD19-CD56-HLA-DR+CD33dim cells. HIV+ Smokers: 89.5±3.5%; HIV+ Non-Smokers: 79.3±8.2%. Statistical significance calculated by Mann-Whitney unpaired test. p-values <0.1 noted on graph, * denotes p-value <0.05

CD16+ expression profile showing the highest frequency (HIV+: 48.3±24.3%; HIV- mean = 33.5±21.6%) (Fig. 21D). Within this CD33dim population the CD14+CD16- expression profile was significantly decreased in HIV+ individuals compared to HIV- individuals (HIV+: 10.3±8.2%; HIV-: 24.2±13.5%; p = 0.026) (Fig. 21D).

The expression of the sialoadhesin CD169 (also known as SIGLEC-1) and the haemoglobin-binding molecule CD163 were examined as these are common scavenger receptors found on tissue macrophages which assist in their function of clearing cellular debris and pathogens. Furthermore, CD169 is not expressed on interstitial macrophages[110], which make up a small proportion of the macrophage pool in the alveolar space, so this marker allowed for assessment of interstitial macrophage frequency. The expression of CD163 has also been implicated in the inflammatory state of the pulmonary space during untreated HIV infection[147,154], and so warranted investigation on these pulmonary cells in the context of long-term successful ART treatment. The majority of AMs co-expressed these markers (HIV+: 68.4±27.4%; HIV-: 74.3±19.5%) (Fig. 22A). There was also a proportion of cells which were CD169+CD163- (HIV+: 25.6±24.8%; HIV-: 16.7±10.3%), and within the HIV+ group smokers showed a tendency towards a larger frequency of CD169+CD163- AMs (Fig. 22A). This loss of CD163 could be attributed to the chronic inflammation in the lung environment of smokers[155]. There was also a small fraction of cells which were CD169-CD163+, described above as interstitial macrophages (HIV+: 2.8±2.1%; HIV-: 5.1±4.6%) (Fig. 22A). The majority of non-AM myeloid cells did not express either CD169 or CD163, regardless of HIV and smoking status, though there was a slight trend towards an increased frequency of CD169+CD163+ cells in HIV+ individuals (data not shown). In the peripheral blood monocytes, however, most cells were CD169- with varying levels of CD163 expression (for

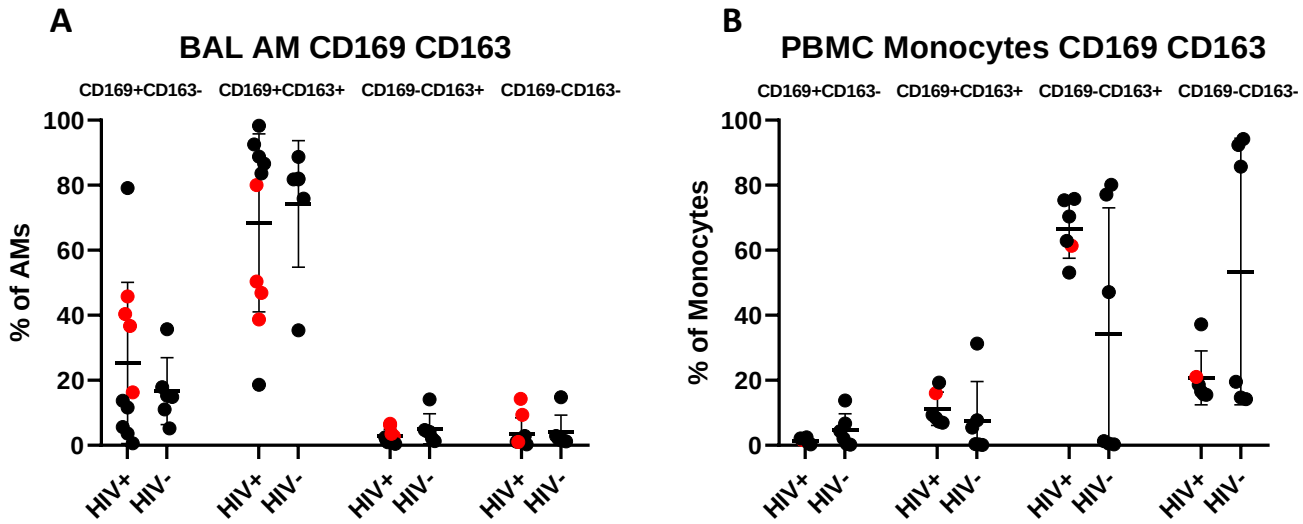

Figure 22. Scavenger receptor expression. Frequency of CD169 and CD163 expression in A. AMs in whole BAL cells or B. monocytes in PBMCs. AMs defined within whole BAL cells as single, live, CD45+HLA-DR+CD206+ cells. Monocytes defined within PBMCs as single, live, myeloid, CD3-CD19-CD56-HLA-DR+CD33+ cells. Statistical significance calculated by Mann-Whitney unpaired test. p-values <0.1 noted on graph, * denotes p-value <0.05.

CD169-CD163+ HIV+: 66.5±8.9%; HIV-: 34.4±38.7% and for CD169-CD163- HIV+: 20.8±8.3%; HIV-: 53. 5±41.0%) (Fig. 22B).

Next we assessed the expression of markers associated with monocyte homing and migration. The chemokine receptors CCR2 and CX3CR1 recruit monocytes to sites of inflammation[153]. CD33 is a sialoadhesin which is expressed only on peripheral myeloid cells, but not mature macrophages[156]. The vast majority of AMs did not express either CCR2 or CD33 (CCR2-CD33- HIV+: 98.3±1.3%; HIV-: 93.0±15.5%) (Fig. 23A). However, within the small fraction of cells expressing CD33,

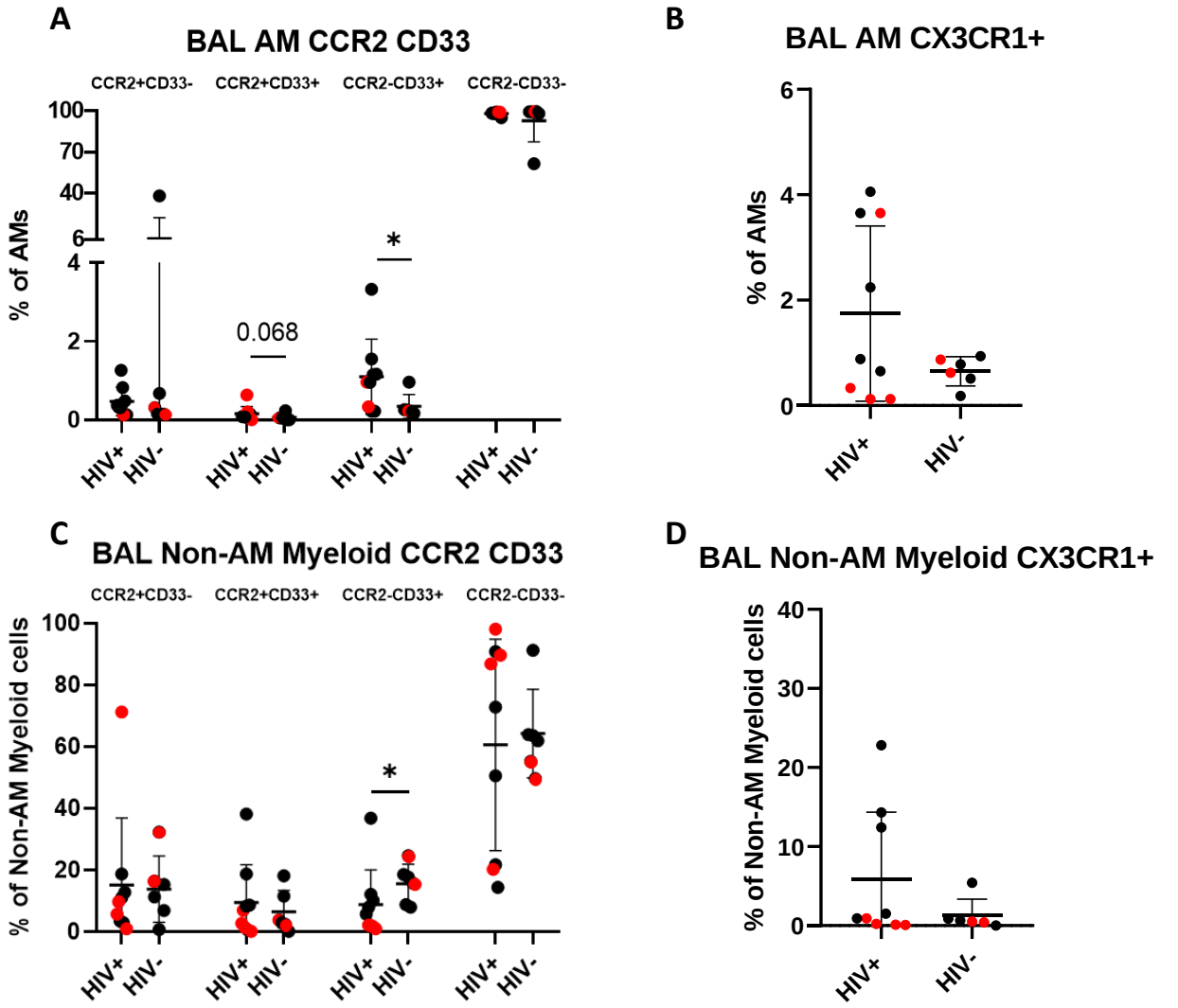

Figure 23. Peripheral myeloid markers in BAL AMs. Frequency of A. CCR2, CD33 and B. CX3CR1 expression in AMs as well as C. CCR2, CD33 and D. CX3CR1 expression in Non-AM myeloid cells. AMs defined within whole BAL cells as single, live, CD45+HLA-DR+CD206+ cells. Non-AM myeloid cells defined within whole BAL cells as single, live, CD45+HLA-DR+CD206- cells. Statistical significance calculated by Mann-Whitney unpaired test. p-values <0.1 noted on graph, * denotes p-value <0.05.

which could be considered those recently migrated from the periphery since this a circulating monocyte marker[156], there was a significant increase in expression of this marker among HIV+ individuals (for CCR2+CD33+ HIV+: 0.15±0.19%; HIV-: 0.06±0.09%; p = 0.068 and for CCR2-CD33+ HIV+: 1.10±0.96%; HIV-: 0.34±0.31%; p = 0.034) (Fig. 23A). Furthermore, when looking at the expression of CX3CR1 in parallel, a similar trend is seen amongst HIV+ individuals toward a higher frequency of CX3CR1+ AMs (HIV+: 1.74±1.66%; HIV-: 0.68±0.28%) (Fig. 23B). These data could suggest an increased infiltration of monocytes from the periphery among AMs from HIV+ donors. Meanwhile, within the non-AM myeloid cells of the BAL, the expression of these markers is slightly increased; interestingly, the frequency of CCR2-CD33+ cells is significantly lower in HIV+ donors compared to HIV+ donors, in contrast to the AMs (HIV+: 8.8±11.2%; HIV-: 15.6±6.3%) (Fig. 23C). Among the non-AM myeloid cells there was no significant difference in the expression of CX3CR1, however (HIV+: 5.9±8.4%; HIV-: 1.3±2.0%) (Fig. 23D).

Further to addressing the question of AM origin, we assessed a marker of stem cells, c-Kit, to see if AMs displayed any stem cell-like properties[157]. In parallel, we looked at the expression of the proliferation marker Ki-67[158] to see if any cells are actively proliferating in this environment. As shown in Fig. 24, only a very small fraction of AMs expressed these markers, while the vast majority of AMs are double negative in both the HIV+ and HIV- groups (HIV+: 96.9±2.7%; HIV-

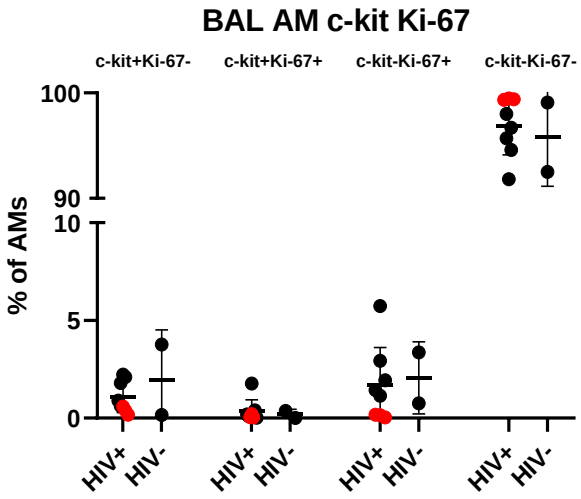

Figure 24. Proliferation and stem-cell like markers in BAL AMs. Frequency of c-Kit and Ki-67 expression in A. AMs and B. Non-AM myeloid cells. AMs defined within whole BAL cells as single, live, CD45+HLA-DR+CD206+ cells. Non-AM myeloid cells defined within whole BAL cells as single, live, CD45+HLA-DR+CD206- cells Statistical significance calculated by Mann-Whitney unpaired test. p-values <0.1 noted on graph, * denotes p-value <0.05.

: 95.8±4.7%) (Fig. 24). Notable, the c-Kit and Ki-67 markers were virtually never co-expressed (for c-Kit+Ki-67+ HIV+: 0.35±0.59%; HIV-: 0.19±0.26%) (Fig. 24).

Finally, based on literature review, the transferrin receptor, CD71, and interleukin 4 receptor, IL-4R, were identified as markers of interest based on their association with particular pathologies, such as pulmonary fibrosis[159] and HIV reservoir maintenance[89]. The expression of CD 71 was robust and consistent between the HIV+ and HIV- cohorts (HIV+: 81.0±12.8%; HIV-: 87.7±12.3%) (Fig. 25A). IL-4R expression was minimal however, and also did not seem to depend on HIV status (HIV+: 4.8±6.4%; HIV-: 2.4±2.4%) (Fig. 25B).

Apart from looking strictly at the immunophenotype of AMs, we briefly examined the expression of the chemokine receptor CCR5 which acts as a co-receptor for HIV. This was done in batch on 4 previously frozen specimens from HIV+ donors and one HIV- donor (data not shown). The mean frequency of CCR5+ AMs was 0.84% with a SD of 0.53%.

3.4 Characterization of Daisy cells as a a novel AM-like cell line

A novel AM-like sub-clone of monocytic THP-1 cells named 'Daisy' cells were used as a substitute for primary AMs from BAL during the COVID-19 pandemic and corresponding interruption to participant recruitment[150]. As this is a novel cell line and has not been characterized in the context of HIV research, we phenotyped these cells to assess their expression profile compared to the THP-1 cells, both with and without mitogenic stimulation using PMA, of various

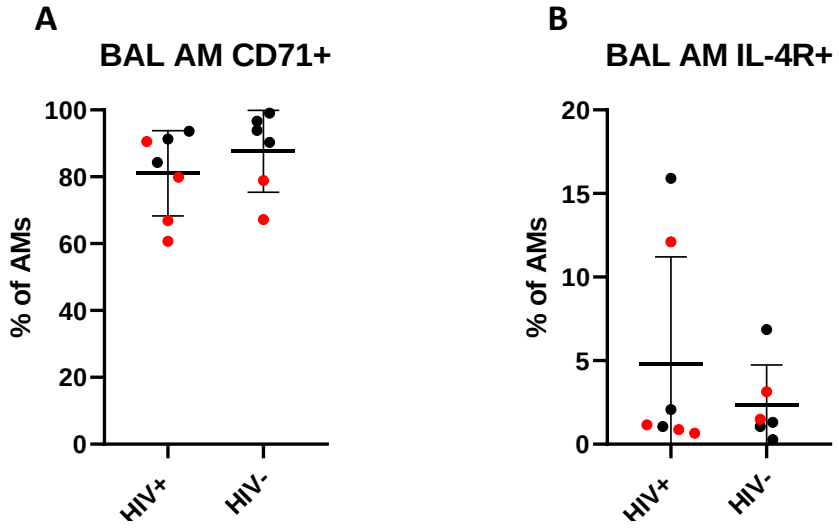

Figure 25. Disease-associated markers in BAL AMs. Frequency of A. CD71 and B. IL-4R expression in AMs. AMs defined within whole BAL cells as single, live, CD45+HLA-DR+CD206+ cells. Statistical significance calculated by Mann-Whitney unpaired test. p-values <0.1 noted on graph, * denotes p-value <0.05.

markers relevant to AM physiology and HIV infection (Fig. 26). Daisy cells showed increased expression of CD14, HLA-DR, CXCR4, CCR5 and CCR2 over THP-1 controls, while levels of CD16, CD163 and CD169 were similar among Daisy cells and PMA-stimulated THP-1 cells (Fig. 26). Notably, for CD206 expression, while fluorescence did shift slightly in Daisy cells compared

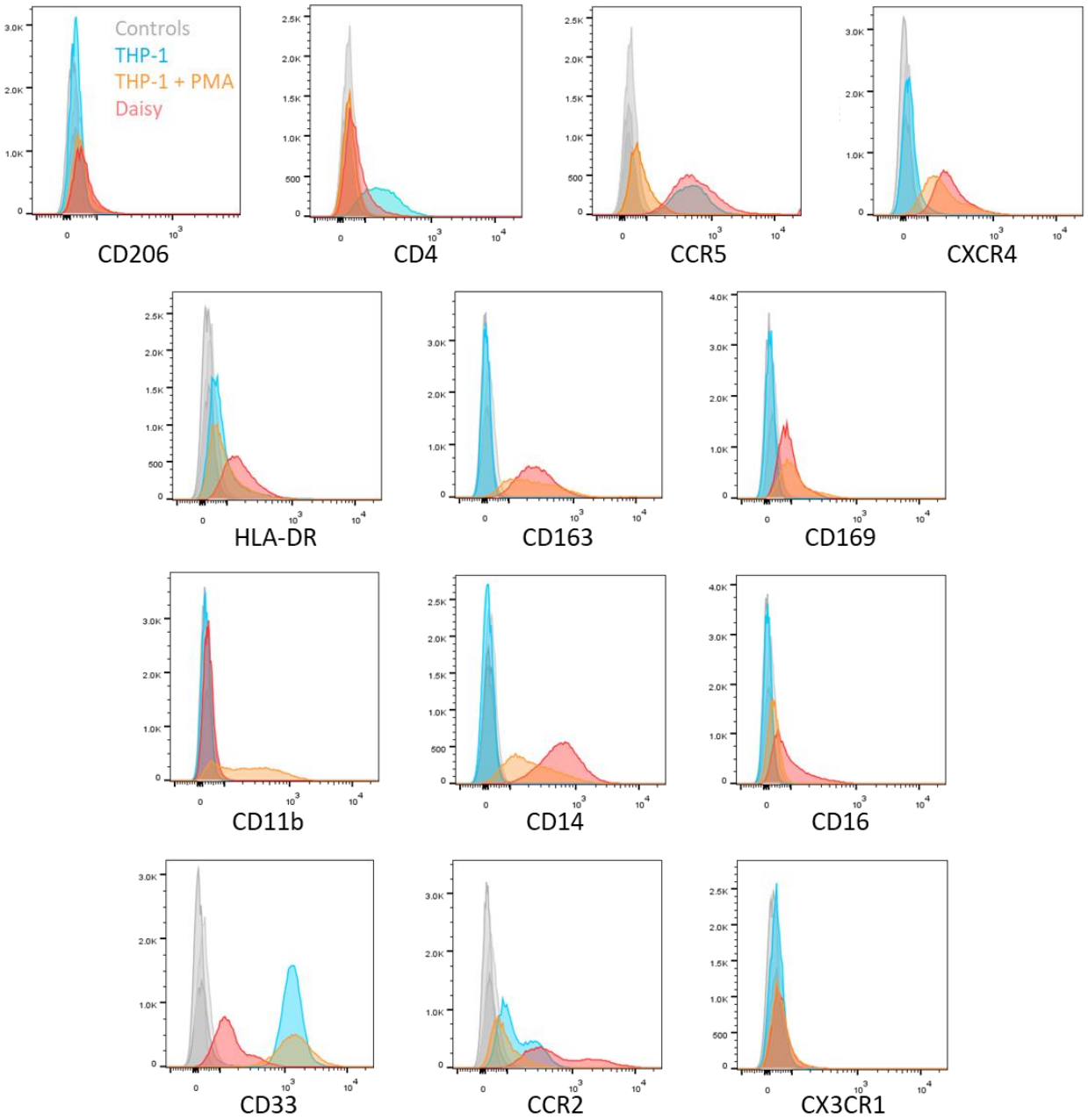

Figure 26. Phenotypic characterization of novel Daisy cell line. Expression of various myeloid markers and HIV receptors and co-receptors on Daisy cells (red peaks), THP-1 cells (blue peaks), and THP-1 cells + PMA (gold peaks). Stimulated cells were incubated with 50nmol PMA for 24h then washed and rested 24h before staining. Unstained cells of each type used as controls (grey peaks).

to THP-1 cells and unstained controls, it was not the drastic shift expected given the previous data which showed a 2-log shift in fluorescence. Therefore, these cells could be considered a tissue macrophage model rather than specificially an AM model. Daisy cells also lacked expression of CD4 and CDllb; a lack of CD4 expression suggests a corresponding lack of susceptibility to HIV infection (Fig. 26).

3.5 *In vitro* infection of MDMs and Daisy cells

Daisy cells were infected in parallel to MDM controls to evaluate their permissiveness to direct HIV infection. Daisy cells and MDMs were infected with 3 HIV strains including an R5 tropic NL4.3-Bal-IRES-HSA; an X4-tropic NL4.3; and a strain pseudotyped with the vesicular stomatitis virus (VSV) envelope, NL4.3-VSV. We used an R5- and an X4-tropic strain as these require differential HIV co-receptor binding. The R5 strain contains a membrane-bound heat stable antigen which is expressed on infected cells and can be detected by a CD24 antibody; in addition, MDMs do not express high levels of CXCR4, so the X4 strain could act as a negative control. The NL4.3-VSV strain was a positive control, confirming that the replication of HIV once in the target cell was not the inhibition of Daisy cell infection, rather it was the binding step. Readout of infection was done evaluating the expression of p24 and, in the case of the NL4.3-Bal-IRES-HSA strain, CD24 (HSA) by flow cytometry at 3 different timepoints (Fig. 27). Daisy cells were only productively infected with the NL4.3-VSV strain (15.7% frequency of p24+ cells), while the R5 tropic NL4.3-Bal-IRES-HSA and X4-tropic NL4.3 strains were unable to produce infection. This experiment was repeated twice with the same results. As the characterization data in Fig. 26 showed, Daisy cells did not express the HIV receptor CD4, so the lack of infection with all but the pseudotyped strain was expected. In the MDM controls, these cells were productively infected by both the R5 tropic strain NL4.3-Bal-IRES-HSA; and the pseudotyped NL4.3-VSV strain but not the X4-tropic strain NL4.3.

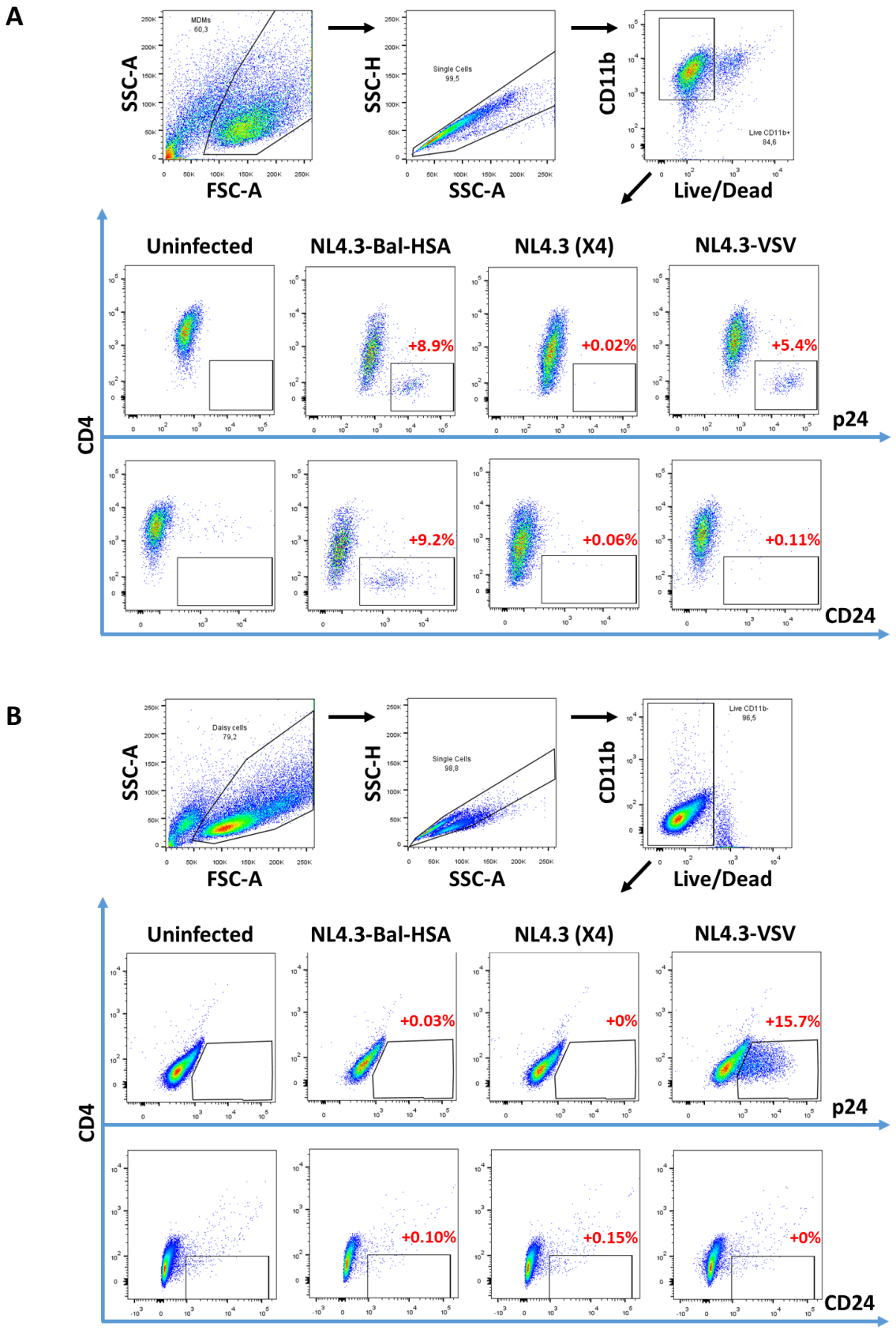
A
MDMs
60,3
SSC-A
FSC-A
Single Cells
99,5
SSC-H
SSC-A
CD11b
Live CD11b+
84,6
Live/Dead
Uninfected
NL4.3-Bal-HSA
NL4.3 (X4)
NL4.3-VSV
+8.9%
+0.02%
+5.4%
+9.2%
+0.06%
+0.11%
CD4
p24
CD24
B
Daisy cells
79,2
SSC-A
FSC-A
Single Cells
98,8
SSC-H
SSC-A
CD11b
Live CD11b-
96,5
Live/Dead
Uninfected
NL4.3-Bal-HSA
NL4.3 (X4)
NL4.3-VSV
+0.03%
+0%
+15.7%
+0.10%
+0.15%
+0%
CD4
p24
CD24

Figure 27. *In vitro* infection of MDMs and Daisy cells. Readout of frequencies of p24 and CD24 (heat stable antigen) expression in **A**. MDMs and **B.** Daisy cells at 2 days post-infection. Representative of 2 experiments. Cells were infected with R5-tropic NL4.3-Bal-IRES-HSA, X4-tropic NL4.3 and pseudotyped NL4.3-VSV strains of HIV. MDMs gated on live, single CD11b+ cells and according to forward scatter and side scatter morphology. Daisy cells gated on live, single CD11b- cells and according to forward scatter and side scatter morphology. Red text denotes increased expression frequency of marker compared to uninfected control.

Chapter 4: Discussion

To explore the contribution of AMs HIV persistence and ongoing susceptibility of PLWH to pulmonary disorders[26,129], we assessed the immune phenotype of these cells. Flow cytometry was performed on fresh cells isolated from BAL fluid obtained from successfully ART-treated PLWH or HIV- participants, with no pulmonary symptoms, in parallel to peripheral blood. We interpreted differences between HIV+ and HIV- individuals, while observing the effect of smoking in tandem. In addition, we attempted to evaluate the origin of AMs to delineate if they were self-renewing embryonically derived cells[112,113] or monocyte-derived macrophages from the periphery. Finally, we aimed to explore whether AMs could be directly infected by HIV, as this would have implications for viral persistence in AMs.

Due to the autofluorescent nature of AMs, particularly from smokers, optimizing the panel of immunophenotyping markers was a considerable challenge. To address this issue, as elaborated in the methods paper of which I am a co-author,[149] we used the AMs themselves as single stain controls rather than compensation beads and we split the markers into two panels to reduce the number of parameters in each, while still allowing a comprehensive panel of markers to be evaluated. Single, live CD45+ cells were gated, and AMs were identified by co-expression of the mannose receptor CD206 and the MHC class II molecule HLA-DR, while non-AM myeloid cells were described as those HLA-DR+ cells which did not express CD206. CD206 has been identified as a canonical marker of human AMs as they express this marker very brightly, as opposed to other tissue-resident macrophages[146]. Within AMs and non-AM myeloid cells, the frequency of expression of all other markers was evaluated and compared between HIV+ and HIV- specimens. Note that the frequency of expression within the parent population is reported, rather than the mean fluorescent intensity (MFI) which is often reported in this context. The reason for this relates to the fact that my samples were stained and acquired individually, with evolving panels and different compensations applied each time depending on the autofluorescence of the cells. As such, the MFI may change significantly from one donor to the next simply due to sample variation and cannot be reported as an assessment of expression level. It quickly became apparent that there is striking heterogeneity in the expression of markers between individuals (Fig. 20). The nature of tissue macrophages is to adapt their varied range of functions to the demands of the niche they inhabit, and my observations are supported by this reputation of plasticity[109]. Even within a particular donor, expression of markers was often not bimodal, but a spectrum displaying diverse concentrations of marker expression among the pool of AMs. Over several years, or even decades,

ART duration and regimens varied amongst HIV+ study participants (Table 4); this is a common limitation in HIV cure research. Also, while HIV+ study participants were all of a similar age, HIV- participants' ages were of a wider range, which could have been a factor in some heterogeneity seen in immune phenotype (Table 4). It is important to note that one criterion for all participants was that they have no pulmonary symptoms or inflammation at the time of bronchoscopy, to ensure any differences observed between cohorts could confidently be attributed to their HIV or smoking status.

At the outset, we examined the expression of markers commonly used to characterize monocytes in the peripheral blood; CD14 is a receptor for LPS and CD16 is an Fcγ receptor. Classical monocytes in the blood express CD14 very brightly and do not express CD16, while through the start of differentiation and maturation, monocytes begin to express CD16 and eventually lose much of their CD14 expression[153]. These CD16+CD14- monocytes are defined as 'non-classical' with more mature, macrophage-like morphology and pro-inflammatory properties[153]. In tissue macrophages, CD14 expression often indicates presence of inflammation, such as in the case of microglia[160] in the brain and intestinal macrophages[161]. These markers were evaluated in AMs and their profile was found to be fitting for a tissue-resident macrophage population; the vast majority of AMs are CD14-CD16+. This follows the expression profile of a mature, differentiated myeloid population, so is not unexpected. Interestingly, smokers appear to cluster together, expressing the highest levels of CD16 within the HIV+ cohort we also characterized those myeloid cells in BAL which were not AMs, defined as HLA-DR+CD206-. This pool of cells would be expected to include some monocytes, but perhaps also some other tissue myeloid cells which simply to not express CD206, such as dendritic cells. In this pool, CD14+ cells are still at a very low frequency. In parallel, we examined the CD14/CD16 profile of monocytes in PBMCs. As expected, the majority were CD14+CD16- classical monocytes, with a smaller population of double positive and CD16 single positive cells. It is worth noting that there was a trend toward a lower frequency of CD14+CD16- monocytes in HIV+ individuals, and a corresponding increase in CD14+CD16+ intermediate monocytes. This could suggest a greater frequency of monocytes in some state of differentiation. When analyzing these data, it became apparent when looking at the CD33 expression on the myeloid cells that there was an intermediate, or 'CD33dim'population which we could identify in all donors, though the population was more distinct in some donors. When this population was examined alone, the expression profile was

markedly different; namely, there was a large increase in the frequency of CD14-CD16+ non-classical monocytes. Further to the trend mentioned above, HIV+ individuals had a significantly lower frequency of CD14+CD16- within this CD33dim population particularly, which could suggest that their peripheral myeloid cells are in a slightly less quiescent state compared to HIV- donors. The identification of this CD33dim population was an interesting observation which, to my knowledge, had not been previously noted.

The sialoadhesin CD169, also known as SIGLEC-1, is a common scavenger receptor found on macrophages, although this marker is not expressed by interstitial macrophages[146]. This 'microheterogeneity' is seen in CD169 within several tissues, including the spleen, bone marrow and epidermis where resident tissue macrophage populations show differing expression levels of this marker[162]. CD163 is another scavenger receptor which binds haemoglobin and is expressed on various myeloid cells[163,164]. Expression of CD163 is present on virtually all tissue macrophages, thought notably, not microglia in the brain[165]. As such, the vast majority of AMs co-express these markers. A small fraction of the AM population were CD169-, which represented interstitial macrophages, while CD163 expression was somewhat more diverse, and within the HIV+ group smokers showed a tendency toward an increased frequency of CD169+CD163- AMs (Fig. 22A). This could suggest some increased propensity for ongoing inflammation, given the anti-inflammatory role of CD163+[147]. Interestingly, a recent study showed that a loss of CD163 expression on AMs occurs in untreated HIV infection[147]. This was associated with the increased inflammation caused by active HIV infection since the CD163+ AMs are considered to play a role in the anti-inflammatory response [163]. In our context of successfully ART-treated individuals, the expression of CD163 appears completely restored, with no statistical difference between frequencies of CD163+ AMs in HIV+ versus HIV- individuals (Fig. 22A). In the peripheral blood, monocytes on the whole did not express CD169. The expression of CD163 varies by donor, however, with some showing fairly high frequency, up to 80%. An increase in CD163 expression has been documented on intermediate CD14+CD16+ monocytes in ART-treated individuals and suggested to be a biomarker of the immune system trying to resolve immune activation[154]. In our cohort, frequency of CD163 expression was consistently high among HIV+ donors (Fig. 22B).

One important question this project sought to address was the origin of AMs and whether the AM pool consists mostly of self-renewing tissue macrophages derived from an embryonic

myeloid precursor, or monocyte-derived macrophages having migrated from the periphery and differentiated. This information is important to inform the design of cure strategies as, if embryonically-derived macrophages are shown to be a true latent viral reservoir which contain replication-competent HIV, clearing virus from these cells to achieve a sterilizing cure would be extremely challenging. Differential penetration of current ARVs in macrophages is a recognized obstacle due to efflux pumps which may eject NRTIs and PIs[107,123]. In addition, virus containing compartments segregate the HIV virions within macrophages and are impenetrable by antibodies, further complicating the challenge of treatment[166]. Proof of self-renewing capacity among HIV-containing macrophages could inform a shift towards functional cure as a more attainable goal than a sterilizing cure, and highlight the need for alternate drug delivery approaches such as the use of liposomes or nanotechnology to access these hard-to-reach tissues[167] To address this question, we included several markers associated with monocyte migration and recruitment. When inflammation occurs, homing of monocytes to sites of inflammation is directed through chemokine signalling. CCR2 is a chemokine receptor commonly associated with classical monocyte homing in response to the CCL2 ligand, while CX3CR1, also known as fractalkine, has been identified as the receptor linked to non-classical monocyte homing in response to the CX3CL1 ligand[153]. As described above, CD33 is another sialoadhesin which is only expressed on peripheral myeloid cells, and so expression of this marker on AMs could give an indication of recent migration and differentiation of monocytes from the periphery. As seen in Fig. 23A, AMs largely do not express CCR2, which is consistent with previous findings suggesting AMs migrate in response to CCL3 rather than CCL2[168]. Within the remaining small fraction of AMs, however, there was a significant increase in the frequency of CCR2-CD33+ AMs in HIV+ individuals compared to HIV-individuals. CD33+ AMs could represent a small population of recently differentiated macrophages derived from monocytes infiltrating from the periphery. Moreover, within AMs, there was also a trend towards increased frequency of CCR2+CD33+ AMs and CX3CR1+ AMs among HIV+ individuals. Taken together, these data may suggest increased infiltration of monocytes from the periphery in HIV+ individuals, which could suggest an increased level of inflammation, despite successful ART. Within non-AM myeloid cells, there was an increased proportion of cells expressing these markers, which was to be anticipated given that this pool is expected to include some monocytes which express these markers (Fig. 23C, D). In contrast to the AM pool of cells, however, within non-AM myeloid cells of HIV+ donors showed a decreased

frequency of CCR2-CD33+ cells (Fig. 23C). Among all these data, smokers are fairly evenly distributed with non-smokers, so more participants would be required to assess if smoking may contribute to the observed differences.

To detect AMs which may have long-lived or stem-cell-like properties, the mast cell growth factor c-Kit was included for analysis[157]. Alongside this, the proliferation marker Ki-67, which is expressed during all but the G0 phase of mitosis and indicates actively dividing cells[158], was examined. By assessing the expression of these two markers together we aimed to determine whether those cells which may have stem-cell-like properties, were proliferating. However, our data show that these two markers are almost never co-expressed in AMs (Fig. 24). Thus, there is a small fraction of cells which are proliferating and an even smaller fraction which express c-Kit, but they are not the same cells. This implies that those cells which may have some stem-cell-like properties are not actively replicating on the whole. As described above, a key feature of tissue resident macrophages is their plasticity, so once monocyte-derived macrophages home to the pulmonary mucosa and take up residence, they become indistinguishable from tissue-resident macrophages. Transcriptomic analysis may be required to fully elucidate the history of these cells, or more complex experimental design, such as studies with lung transplant cell tracing[115].

I also evaluated the expression of the transferrin receptor CD71 and interleukin-4 receptor IL-4R on AMs. A loss of CD71 on AMs is associated with pulmonary fibrosis progression[159] while IL-4R expression was increased on urethral tissue macrophages identified as reservoirs of HIV in ART-treated individuals[89]. In my hands, however, we did not see any significant differential expression of these markers on AMs, either based on HIV or smoking status. This goes to show the differences in marker expression based on the niche of tissue macrophages. CD71 expression was robust and consistent between groups, as expected for tissue macrophages, while IL-4R expression was limited (Fig. 25). One interesting point of observation, however, is that smokers tended exhibit the lower frequencies of CD71+ AMs, both within the HIV+ and HIV- groups. More samples would be needed to ascertain whether this is a true trend rather than an artifact, but this trend would be consistent with previous studies that show decreased expression of CD71 in current smokers[169].

Finally, we aimed to address whether AMs could be directly infected by HIV or whether they were infected solely via phagocytosis, as has been previously shown[120]. To assess this

question, we examined the expression of the HIV co-receptor CCR5 which is required for infection with an R5-tropic virus.

Originally, the next step following optimization was to validate the infection in AMs isolated from HIV- participants to determine if it was possible to directly infect these cells, as was the case with MDMs. However, due to the onset of the COVID-19 pandemic, participants were no longer allowed to come to the clinic to participate in research studies for their safety, so we tried to find an alternative model for the *in vitro* infection protocol. As an alternative, a recent paper described a novel human alveolar macrophage-like cell line named 'Daisy' which was a sub-clone of a commonly used macrophage precursor cell line, THP-1[150]. This group identified several characteristics of these Daisy cells which seemed to lend them an AM-like phenotype, such as increased phagocytic capacity, adherent monolayer cell morphology, and high expression of CD206. we obtained a vial of these cells and validated some of the markers the previous group had examined in the Daisy cells, THP-1 cells and THP-1 cells stimulated with PMA, as well as assessing markers relevant to our work in the context of HIV, such as CD4, CCR5 and CXCR4 (Fig. 26). While some of the markers showed similar expression levels seen in the previous data, there was a marked difference in the CD206 expression. Our data show that while Daisy cells do express CD206, it is not at the high levels described by the group. Furthermore, there was minimal expression of the HIV receptor, CD4, which negated their potential worth as a model for HIV infection despite robust expression of the HIV co-receptors CCR5 and CXCR4 (Fig. 26). However, Daisy cells' comparable or increased expression levels of CD163, CD169, CD16 and HLA-DR compared to PMA-stimulated THP-1 cells, the alternative *in vitro* model of macrophages, confirm that these cells do exhibit a mature macrophages phenotype without the need for stimulation. This exemplifies them as a useful tool for macrophage research overall, in particular given their adherent phenotype, though we would be cautious to specifically class them as AM-like given their minimally increased expression of CD206 over THP-1 cells. Daisy cells are an adherent, macrophage-like cell line which is a tool not currently available; the closest cell line candidates are the pro-monocytic cell lines THP-1, described above, and U937, both of which require stimulation of some kind to induce differentiation[170] .

I carried out the *in vitro* infection on the Daisy cells, in parallel to MDMs as a control, using 3 strains of HIV. The first was the NL4.3-Bal-IRES-HSA mentioned above allowing for

detection of infected cells by surface expression of murine CD24. In parallel, we infected Daisy cells and MDMs with another NL4.3 strain which is X4 tropic and a third NL4.3 strain which was pseudotyped with the vesicular stomatitis virus envelope VSV-G. The VSV envelope allows the virus to bypass the need for CD4 and CCR5 binding to enter the cell and enter by clathrin-mediated endocytosis[152] . Following this, the HIV can form a single round of replication. As predicted by the lack of CD4 expression, the Daisy cells were not infected by either the NL4.3-Bal-IRES-HSA strain or the X4-tropic NL4.3 strain, while 15% of cells were p24 positive following infection with the VSV-G pseudotyped virus (Fig. 27B). This confirms that the cells can replicate the virus, but the lack of CD4 expression prevents virus binding and entry into the cells. While these cells may still prove a useful *in vitro* macrophage model, they could not step in for AMs in this context. Once patient recruitment resumes and this optimized infection protocol can be applied to primary AMs, the permissiveness to these cells to direct infection can be explored and this could have further implications for the direction if HIV reservoir research.

One of the most significant challenges facing any macrophage research is the difficulty involved in accessing samples of relevant tissues. Even in the case of fresh AMs isolated with relative ease from BAL, we have to consider the artificial environment of cell culture and how manipulation of these cells *in vitro* may affect their behaviour when we want to make inferences about their permissiveness to infection, for example. Many studies of tissue macrophages require invasive biopsies to retrieve primary macrophages from anatomical sites such as lymph nodes, liver, or colon. In the case of immune privileged sites such as the brain[84 97],access to specimens may only be possible through the 'rapid research autopsy' method whereby a terminally ill individual indicates their desire to donate their body to medical research upon their death. This is exemplified by the 'Last Gift' study at the University of California San Diego[171] and this model has recently been adopted by the Canadian HIV Cure Enterprise (CanCURE) to develop a post-mortem HIV tissue biobank[172]. While BAL is far less invasive than any of these interventions, the procedure is still complicated logistically and expensive, with risks of fever and bleeding[149]. As such, mouse models of AMs are commonplace and offer significant advantages. These studies allow for greater control and reproducibility of experiments, and more complex questions can be addressed, such as fate-mapping using Cre/lox crossing of mice to follow the fate of embryonic myeloid progenitors[113] or generating a Myeloid-Only Mouse model lacking T cells to prove the ability of HIV infection to progress independently of T cells[85]. These experimental designs are

elegant and allow the research community to probe questions that could never be addressed in humans, but mouse models are far from representative of what is happening in the physiology of human infection and many markers used to study AMs in mice are different from those expressed on human AMs. Non-human primate (NHP) models are often used as the last step before trialling a new treatment in humans given their similarity, especially in the context of HIV infection. SIV follows a very similar natural history to HIV in specific NHPs, including rapid seeding of viral rservoirs[75,173]. Interestingly, lung damage can be attributed to preferential destruction of interstitial macrophages rather than AM in the case of SIV infection[174]. Another study showed increased expression of inflammatory chemokines in the lungs during systemic HIV infection[175] which is corroborated by data generated studying the hilar lymph nodes draining from the lungs[176]. Going one step further, the advent of research with SIV-HIV chimeric viruses, or 'SHIVs' allow for an even more relevant model of human HIV-related disease progression in NHPs[177]. One such study of virus-associated pneumonia demonstrated the recruitment of macrophages to the pulmonary space and viral replication in lung[178] macrophages. Despite the elegance of NHP models, however, any findings from these studies must be validates in humans; a recent study showing increased expression of the exhaustion marker PD-1 in AMs from chronically SIV-infected rhesus macaques also demonstrated high levels of CD14 on these AMs, which is in contract to human AMs which express low levels of CD14. As such, these various models must be employed in a complementary manner to further research in this field in a comprehensive way.

Conclusion and Future Directions

Overall, for many markers indicating macrophage function and activation there is no observable difference between HIV+ and HIV- individuals, proving the resolution of some of the disruptions to the pulmonary immune environment that occur upon HIV infection. This is in contrast to the decrease in CD206 and CD163 expression and increase in HLA-DR expression seen in untreated infection, showing that ART restores some of these immune perturbations[147]. Differences in expression of myeloid homing markers on AMs from HIV+ and HIV- individuals, however, indicate there may be some increased infiltration of monocytes from the periphery differentiating into AMs in HIV+ participants. This is suggested by the increase in CCR2-CD33+ AMs among HIV+ individuals and the trend toward an increase in CX3CR1+ AMs, expressed by more mature, macrophage-like CD16+ monocytes in the periphery. Since tissue-resident AMs are able to renew their pool of cells without replenishment from the periphery[113], this suggests that the infiltration could be in response to some form of immune activation in the pulmonary space. As alluded to previously, if it can be shown that replication-competent virus resides in AMs which are in the pool of self-renewing embryonically-derived cells, the implications for future HIV cure strategies would be significant.

Smoking also appeared to influence the expression, or lack thereof, of certain markers. Within the HIV+ group, smokers appeared to have lower frequencies of CD163 expression, possibly reflecting chronic inflammation present in the pulmonary mucosa of smokers, as CD163 plays an anti-inflammatory role on tissue macrophages[147]. A tendency for lower frequency of CD71+ AMs was also seen in smokers of both HIV+ and HIV- groups, which fits with previous findings that this marker is lost in the AMs of smokers[169].

While phenotyping of AMs can give us insight into the action and behaviour of these cells, there is information within the cell that can also be informative. Transcriptomic studies of these cells could provide insights into their physiology and how they may change in the context of ART-treated HIV infection and smoking. AMs from HIV+ and HIV- donors could be compared as was done here, or AMs from an HIV- individual could be assessed before and after *in vitro* infection to evaluate differences that occur in acute HIV infection. Indeed, when comparing untreated HIV+ smokers with HIV- non-smoker, Logue et al.[155] reported an increase in chitinase 1 expression in the HIV+ smokers. In the liver, transcriptomic analysis identified the *Timd4* gene as a temporary

indicator or embryonic origin among Kupffer cells[179]. To address the question of the ontogeny of AMs, transcriptomic methods such as these may be required since macrophage plasticity means their surface expression is more likely to reflect their current niche and the corresponding demands on the cells, rather than their origin.

Finally, while the original project aim to test the susceptibility of AMs to direct HIV infection was not achievable due to the COVID-19 interruption, an MDM model for *in vitro* infection was developed and can be applied to AMs once primary cell collection becomes available again. Meanwhile, the Daisy cell line was characterized for potential use as an AM substitute. These cells express many markers of mature macrophages, but do not express CD4, the receptor of HIV. As expected, therefore, when infected with an R5-tropic, X4-tropic and VSV-pseudotyped strain of HIV, only the latter was able to infect these cells, confirming that Daisy cells, while validated as an adherent macrophage cell line, cannot be used in the context of HIV infection research.

Further to this aim of exploring AM permissiveness to HIV infection, I assisted with the generation of HIV strains to assess the indirect infection of AMs, via phagocytosis of infected T cells. We generated an X4-tropic NL4.3-GFP which could be used to infect T cells that are then co-cultured with AMs to observe engulfment the T cells. Another approach would be to prime cells with Vpx prior to infection, which disrupts the action of SAMHD1, restoring the reverse transcriptase activity and potentially increasing infection efficiency in AMs. I also assisted in generating a virus-like particle containing Vpx to address this question in the future. In the context of exploring tissue macrophages as cellular HIV reservoirs as described above, Ganor *et al.*[89] identified replication-competent virus in urethral macrophages from ART-treated PLWH using a quantitative viral outgrowth assay (QVOA) adapted for the smaller cell numbers associated with primary tissue macrophage sources. In contrast, however, groups studying liver macrophages[91] and colon monocytes and dendritic cells[90] from ART-treated PLWH were not able to reactivate replication-competent virus from these compartments, despite presence of HIV DNA. It is clear, therefore, that each anatomical site must be investigated individually. The natural next step is to perform a QVOA on AMs from BAL and evaluate whether the HIV DNA that is present in these cells constitutes a true latent viral reservoir that can be reactivated, such as in the context of ART interruption.

As mentioned earlier, infection with HIV has been identified as an independent risk factor for development of COPD, regardless of smoking status. However, it is well-documented that PLWH are more likely to be smokers[135], and therefore have increased risk for acquisition of COPD on both these fronts. Due to the nature of the lungs' interaction with the exterior environment, they have a rich microbiome which may be perturbed by certain disease states or chronic inflammation. It has also been shown that when microbes from the upper airways are enriched in the lower airways, there is a correlation with increased lung inflammation[180]. The ubiquitous presence of these microbes and other environmental antigens like pollutants and tobacco mean AMs are susceptible to undergo oxidative stress. HIV infection and ARVs can both increase reactive oxygen species (ROS)[181] and there is clear evidence of increased oxidative stress in the BAL of those with COPD[182]. Understanding the environment with which AMs are interacting can give us further insight into how they may be impaired in the context of HIV infection or COPD. Indeed, future work planned in our group will investigate the relationship between the pulmonary HIV reservoir and the severity of COPD in PLWH.

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