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IMMUNOMETABOLIC REPROGRAMMING DURING SUPPRESSIVE HIV-1 INFECTION

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Immunometabolic reprogramming during suppressive HIV-1 infection

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By

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This thesis is dedicated to the memory of my late grandmother, Singalill Svensson

ABSTRACT

Since the implementation of antiretroviral therapy (ART), infection with human immunodeficiency virus type-1 (HIV-1) has been transformed into a chronic lifelong condition. The main obstacle for a HIV-1 cure is the persistence of latently infected cells in viral reservoirs. The viral endurance can instigate detrimental changes on the function and activity of immune cells, creating a chronic inflammatory environment in people living with HIV-1 (PLWH) on successful long-term suppressive antiretroviral therapy ($PLWH_{ART}$). The continuous activation of immune cells may lead to an earlier onset of age-related diseases. Immunometabolism is an emerging field that studies how metabolic reprogramming has an impact on the activation, differentiation, and function of immune cells. Given that these underlying processes are likely to contribute to chronic inflammation in PLWH, the overall aim of this thesis was to evaluate how immunometabolism is reprogrammed during "controlled" HIV-1 infection, either by ART in $PLWH_{ART}$ or in PLWH with natural control of infection, elite controllers ($PLWH_{EC}$).

In **paper I**, we integrated proteomic and transcriptomic data to investigate features distinct to the $PLWH_{EC}$ phenotype in a male cohort. We identified dysregulated hypoxia inducible factor (HIF) signalling and altered metabolism as unique characteristics of the male $PLWH_{EC}$ phenotype. As controlled HIV-1 infection still induce changes in the immune system we aimed to compare differences in the immune phenotype between $PLWH_{EC}$ and $PLWH_{ART}$ and its relation to HIV-1 persistence in **paper II**. We identified a unique phenotype of decreased CCR6 expression on $CD4^+$ and $CD8^+$ T cells in $PLWH_{EC}$ compared to $PLWH_{ART}$ and healthy controls (HC). Additionally, the $CD4^+CCR6^+$ cells exhibited a proteomic profile indicative of increased sensitivity towards cell death mechanisms in $PLWH_{EC}$ compared to $PLWH_{ART}$. A reduced proportion of integrated HIV-1 DNA in the reservoir of $PLWH_{EC}$ was found, although no difference in the amount of intact provirus. Continuing our evaluation of differences between $PLWH_{EC}$ and $PLWH_{ART}$ we performed metabolo-transcriptomic analysis to understand and infer changes on a multisystem level in **paper III**. We detected a system level metabolic aberration mainly revolving around OXPHOS in $PLWH_{ART}$ compared to $PLWH_{EC}$. Using pharmacological modulation, we identified how this dysregulation of OXPHOS possibly affects HIV-1 reservoir dynamics and the immune senescence profile. Furthermore, to understand how HIV-1 chronicity affects long-lasting metabolic flexibility and adaptation we conducted plasma metabolomics to understand alterations during suppressive ART in a Swedish cohort in **paper IV**. We also aimed to characterize the cell populations that mainly contribute to changes in the metabolic environment. We detected aberrant energy metabolism in $PLWH_{ART}$, mainly revolving around the tricarboxylic acid cycle and amino acid synthesis. Cell-type specific evaluation showed that the main metabolic alterations occurred on monocytic cell populations, and that $PLWH_{ART}$ exhibited dysregulated chemokine receptor expression of CCR2, CCR5, and CX3CR1 on myeloid cell lineages. In **paper V**, we wanted to evaluate if the altered metabolic environment was consistent on a global scale using two cohorts from low and middle-income countries (namely, Cameroon and India) using plasma metabolomics. We detected a dysregulation of amino acid metabolism and a switch towards glutaminolysis during long-term suppressive ART.

In summary, the research covered in this thesis illuminates the importance of metabolic reprogramming during HIV-1 persistence in PLWH with controlled infection.

LIST OF SCIENTIFIC PAPERS

I. **Sara Svensson Akusjärvi***, Anoop T Ambikan*, Shuba Krishnan, Soham Gupta, Maike Sperk, Ákos Végvári, Flora Mikaeloff, Katie Healy, Jan Vesterbacka, Piotr Nowak, Anders Sönnerborg, and Ujjwal Neogi. *Integrative proteo-transcriptomic and immunophenotyping signatures of HIV-1 elite control phenotype: A cross-talk between glycolysis and HIF signaling.* iScience, 2022 Jan 21; 25(1):103607

II. **Sara Svensson Akusjärvi,** Shuba Krishnan, Bianca B. Jütte, Anoop T Ambikan, Soham Gupta, Jimmy Esneider Rodriguez, Ákos Végvári, Maike Sperk, Piotr Nowak, Jan Vesterbacka, J. Peter Svensson, Anders Sönnerborg, and Ujjwal Neogi. *Peripheral blood $CD4^{+}CCR6^{+}$ compartment differentiates HIV-1 infected or seropositive elite controllers from long-term successfully treated individuals*, Communications Biology, 2022 April 13; 5(1):357

III. Anoop T Ambikan*, **Sara Svensson Akusjärvi***, Shuba Krishnan, Maike Sperk, Piotr Nowak, Jan Vesterbacka, Anders Sönnerborg, Rui Benfeitas, and Ujjwal Neogi. G*enome-scale metabolic models for natural and long-term drug-induced viral control in HIV-infection,* Accepted, Life Science Alliance

IV. **Sara Svensson Akusjärvi**, Shuba Krishnan, Anoop T Ambikan, Flora Mikaeloff, Sivasankaran Munusamy Ponnan, Jan Vesterbacka, Magda Lourda, Piotr Nowak, Anders Sönnerborg, and Ujjwal Neogi. *Monocyte driven system-level inflammatory and immunometabolic dysregulation during prolonged successful HIV-1 treatment,* Manuscript format

V. Flora Mikaeloff, **Sara Svensson Akusjärvi**, George Mondinde Ikomey, Shuba Krishnan, Maike Sperk, Soham Gupta, Gustavo Daniel Vega Magdaleno, Alejandra Escós, Emilia Lyonga, Marie Claire Okomo, Claude Tayou Tagne, Hemalatha Babu, Christian L. Lorson, Ákos Végvári, Akhil C. Banerjea, Julianna Kele, Luke Elizabeth Hanna, Kamal Singh, João Pedro de Magalhães, Rui Benfeitas, and Ujjwal Neogi. *Trans cohort metabolic reprogramming towards glutaminolysis in long-term successfully treated HIV-infection,* Communications Biology, 2022 Jan 11;5(1):27

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II. Marco Gelpi, Flora Mikaeloff, Andreas D Knudsen, Rui Benfeitas, Shuba Krishnan, **Sara Svensson Akusjärvi**, Julie Høgh, Daniel D Murray, Henrik Ullum, Ujjwal Neogi, and Susanne D Nielsen. *The central role of the glutamate metabolism in long-term antiretroviral treated HIV-infected individuals with metabolic syndrome.* Aging (Albany NY), 2021 Oct 11; 13(19): 22732-22751

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IV. Maike Sperk, Flora Mikaeloff, **Sara Svensson Akusjärvi**, Shuba Krishnan, Sivasankaran Munusamy Ponnan, Anoop T Ambikan, Piotr Nowak, Anders Sönnerborg, and Ujjwal Neogi. *Distinct lipid profile, low-level inflammation, and increased antioxidant defense signature in HIV-1 elite control status.* iScience. 2021 Jan 28; 24(2):102111

V. Sofia Appelberg, Soham Gupta, **Sara Svensson Akusjärvi**, Anoop T Ambikan, Flora Mikaeloff, Elisa Saccon, Ákos Végvári, Rui Benfeitas, Maike Sperk, Marie Ståhlberg, Shuba Krishnan, Kamal Singh, Josef M Penninger, Ali Mirazimi, and Ujjwal Neogi. *Dysregulation in Akt/mTOR/HIF-1 signaling identified by proteo-transcriptomics of SARS-CoV-2 infected cells.* Emerging Microbes and Infections. 2020 Dec; 9(1):1748-1760

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VII. Hemalatha Babu, Anoop T Ambikan, Erin E Gabriel, **Sara Svensson Akusjärvi**, Alangudi Natarajan Palaniappan, Vijila Sundaraj, Naveen Reddy Mupanni, Maike Sperk, Narayanaiah Cheedarla, Rathinam Sridhar, Srikanth P Tripathy, Piotr Nowak, Luke Elizabeth Hanna, and Ujjwal Neogi. *Systemic Inflammation and the Increased Risk of Inflamm-Ageing and Age-Associated Diseases in People Living With HIV on Long Term Suppressive Antiretroviral Therapy.* Frontiers in Immunology. 2019 Aug 27; 10:1965

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LIST OF ABBREVIATIONS

αKG	α-ketoglutarate
3TC	Lamivudine
AA	Amino acid
Acetyl-CoA	Acetyl coenzyme A
Ab	Antibody
ADCC	Antibody dependent cell mediated cytotoxicity
AIDS	Acquired immunodeficiency syndrome
Akt	Protein kinase B
AMPK	AMP-activated protein kinase
APOBEC3G	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G
ART	Antiretroviral therapy
AZT	Zidovudine
c-Myc	Cellular myelocytomatosis oncogene
CCM	Central carbon metabolism
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CM	Classical monocytes
CMV	Cytomegalovirus
CNS	Central nervous system
CRP	C-reactive protein
CTL	Cytotoxic T lymphocyte
CXCR	C-X-C chemokine receptor
DC	Dendritic cell
DCQ	Digital cell quantification
ddPCR	Digital droplet polymerase chain reaction
DEG	Differentially expressed genes
EFV	Efavirenz
EPIC	Estimating the proportions of immune and cancer cells
FACS	Fluorescence-activated cell sorting

FAO	Fatty acid oxidation
FAS	Fatty acid synthesis
FBA	Flux balance analysis
FDC	Follicular dendritic cell
GALT	Gut associated lymphoid tissue
Glut1	Glucose transporter 1
GSEA	Gene set enrichment analysis
GSH	Reduced glutathione
GSMM	Genome-scale metabolic model
HC	HIV-1 negative healthy control
HIF	Hypoxia inducible factor
HIV-1	Human immunodeficiency virus type 1
HLA	Human leukocyte antigen
IC-qPCR	Internally controlled quantitative polymerase chain reaction
IFN	Interferon
IL	Interleukin
IPDA	Intact proviral DNA assay
IM	Intermediate monocytes
INSTI	Integrase strand transfer inhibitor
LPS	Lipopolysaccharide
LRA	Latency reversal agent
LTR	Long terminal repeat
MCT1	Monocarboxylate transporter 1
MDSC	Myeloid derived suppressor cells
MetS	Metabolic syndrome
MFI	Median fluorescence intensity
MNP	Mononuclear phagocytes
mTOR	Mammalian target of Rapamycin
NCM	Non-classical monocytes
NK	Natural killer
NNRTI	Non-nucleoside reverse transcriptase inhibitor

NRTI	Nucleoside reverse transcriptase inhibitor
OXPHOS	Oxidative phosphorylation
PBMC	Peripheral blood mononuclear cell
PCA	Principal component analysis
PD-L1	Programmed cell death 1 ligand 1
PI	Protease inhibitor
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase c
PLWH	People living with HIV-1
$PLWH_{ART}$	People living with HIV-1 on suppressive ART
$PLWH_{EC}$	Elite controllers
$PLWH_{Naive}$	Therapy naïve people living with HIV-1
PPP	Pentose phosphate pathway
PrEP	Pre-exposure prophylaxis
PRR	Pattern recognition receptor
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
sCD14	Soluble CD14
sCD163	Soluble CD163
t-SNE	T-distributed stochastic neighbour embedding
TCA	Tricarboxylic acid cycle
TCR	T cell receptor
TDF	Tenofovir disoproxil fumarate
Th	T helper
TNF	Tumour necrosis factor
Treg	Regulatory T cell
TWEAK	Tumour necrosis factor (ligand) superfamily, member 12
UMAP	Uniform manifold approximation and projection
VL	Viral load
WHO	World Health Organisation
xCT	Cysteine/glutamate transporter

1 INTRODUCTION

Human immunodeficiency virus 1 (HIV-1) is a retrovirus that emerged into the public's view in the early 1980's [1]. Since then, the global dissemination of HIV-1 has affected all corners of the world. It was only after the implementation of combination antiretroviral therapy (ART) in the middle of the 1990's that HIV-1 was transformed from a deadly disease into a chronic lifelong infection [2]. Unfortunately, the global economic distribution and limited availability of ART still contributes to a large fraction of newly infected cases per year (WHO estimated 1.5 million in 2020) and deaths due to HIV-related causes (WHO estimated 680 000 in 2020) [3]. Successful ART suppresses viral replication to undetectable levels in the body. However, a fraction of immune cells harbouring latent HIV-1 persists during ART, comprising the major HIV-1 reservoir. These latent cells establish early on during HIV-1 infection and so far, no identifiable markers distinguishing these cells have been found. As a result, HIV-1 persistence contributes to a chronic inflammatory environment in people living with HIV-1 (PLWH) on suppressive therapy ($PLWH_{ART}$). Metabolic reprogramming is one of the main mechanisms steering and mediating modulation of inflammatory responses in cells. Lately, immunometabolism during viral infections has been described to contribute to the dysregulation of immune cell functions. Even as HIV-1 is a treatable condition, chronic inflammation and persisting virus can result in earlier onset of age-related diseases. Although a large amount of research has been performed during the last decades, further studies are still needed to galvanize research for an HIV-1 cure.

1.1 HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 (HIV-1)

HIV-1 belongs to the family *Retroviridae* in the genus *Lentiviruses*. Even though it first received global awareness in the 1980's, researchers today have traced the first human cases as far back as 1956 in Kinshasa. The origin has been traced to chimpanzees and gorillas living in the region between Cameroon and Congo [4]. In Sweden, the HIV-1 epidemic was introduced in 1979 [5]. As an RNA virus, the complete genomic transcript of HIV-1 is approximately 9.2 kb unspliced. The encoded viral proteins are divided into the structural proteins *gag* (matrix (p17), capsid (p24), p6, and nucleocapsid) and *env* (the surface molecules gp120 and gp41). Additionally, it contains the *pol* (protease, reverse transcriptase, and integrase) for enzymatic functions, essential regulatory elements (*tat* and *rev*), and the accessory regulatory proteins for viral assembly (*nef*, *vpr*, *vif*, and *vpu*) [6]. For entry into a cell, HIV-1 is dependent on CD4 as the main receptor [7, 8] and one of the chemokine receptors CCR5 [9-13] and/or CXCR4 [14] as co-receptors. Interactions of the env proteins with the cell surface receptors lead to a structural change ultimately resulting in fusion of the viral and cell surface membranes. As a retrovirus, HIV-1 utilizes the host cell machinery in combination with viral reverse transcriptase to revert the two RNA strands into double stranded DNA copies. With the help of viral encoded integrase, the viral DNA can be inserted into the cellular genome where it is either transcribed, completing the phase of lytic viral production, or remains transcriptionally inactive, in a latent state [6]. HIV-1 is thus highly dependent on the host cellular machinery for propagation, and the activity of the HIV-1 promoter is tightly associated with the transcriptome of the host cell [15]. Transcriptional activation occurs through upstream signals, inducing formation and activation of the transcriptional initiation complex. Recruitment of tat, the viral encoded transactivator

protein, removes negative elongation factors thereby resulting in complete transcription of the viral genome [16]. This results in a tat-mediated elongation which is required for full HIV-1 transcription. Alternative mechanisms of tat-independent basal HIV-1 transcription have also been described, but the resulting extent of fully spliced mRNA and virus production is not fully understood [17]. The functional process of HIV-1 infection and integration has been well delineated, and therapies targeting the different stages of viral replication are available. Still, the biological consequences of long-term infection and potential strategies for an HIV-1 cure remain to be elucidated.

1.1.1 People living with HIV-1 (PLWH)

Establishment of HIV-1 in the human body results in the rapid production of a large amount (10^{10}) of viral copies per day. The initial phase, usually within one to four weeks after exposure, is referred to as the primary (acute) phase when most individuals experience different kinds and degrees of clinical presentations (e.g., fever, rash, and sore throat). Even so, a substantial number of individuals have no or very mild symptoms [18]. The primary phase is initially associated with a very high viral load (VL) and rapid $CD4^+$ T cell decline [19]. After 4-6 weeks, the disease progresses into the chronic phase where the body's immune system suppresses viral replication, but viral escape can lead to detrimental effects. Untreated HIV-1 infection is the causative factor for the development of acquired immunodeficiency syndrome (AIDS). Progression of the disease to AIDS occurs continuously in most untreated individuals, with different rates ranging from one to ten years [20]. AIDS is a clinical definition of when the individual exhibits any of the AIDS defining conditions and in some counties a cut-off where $CD4^+$ T cell count decline below 200 cells/mm^3 blood [21]. However, today in Sweden the AIDS-diagnosis is not used in the clinic since it has no consequences for the individual, treatment, or society. This progressive depletion renders the immune system ineffective at defending the body against pathogens, thereby resulting in higher susceptibility to opportunistic infections and the development of some cancers. Untreated people living with HIV-1 ($PLWH_{naïve}$) are living with a constantly activated immune system and are at high risk of transmitting the virus to others. However, todays efficient ART shuts down the viral replication to undetectable levels in blood plasma and eliminates the contagiousness [22]. As a preventive strategy, pre-exposure prophylaxis (PrEP) has been introduced to reduce transmission events in high-risk populations when administered daily, or at selected occasions [23-26]. If infected, achieving control of viral replication is imperative to improve the health, well-being, and quality of life of PLWH.

1.1.2 Natural control of HIV-1 infection in elite controllers ($PLWH_{EC}$)

There are a few individuals capable of controlling the virus in the absence of ART. These individuals are referred to as PLWH with elite control ($PLWH_{EC}$) and represent <0.5% of all infected individuals [27-31]. Even as no coherent definition of the state exists across cohorts, $PLWH_{EC}$ are generally defined by a VL below the detectable level of sensitive RNA assays, and a maintenance of high $CD4^+$ T cell counts. Research has strived to utilize the phenotype of natural control as a model for immunity to resolve mechanisms underlying disease progression. Despite these efforts, exactly what renders the body capable of this natural control is not fully understood.

Early studies suggested that natural control of HIV-1 is mediated by high integration of virus with impaired replication capacity [32-35]. Attenuation of the virus was described by mutations in both *env* and the viral accessory proteins [36-38]. One recent study also showed how three $PLWH_{EC}$ with natural control for more than 25 years had low viral genetic diversity, possibly caused by the high frequency of replication-impaired integrated virus and low-level inflammation [39]. Even so, replication competent virus has been isolated from $PLWH_{EC}$ [40-42], thereby disproving this hypothesis as the sole mechanism of natural control of infection. Nonetheless, replication competent virus is still not recovered from all $PLWH_{EC}$, and one study also showed how cytotoxic $CD8^+$ T cell responses were higher in individuals from which the virus could be isolated [43]. Additionally, researchers have shown that the integrated provirus has an intact structure and is mainly localized in chromatin regions carrying repressive histone modifications in $PLWH_{EC}$ [44]. Another proposed factor for natural control is HIV-1 specific $CD8^+$ T cell which have higher activation and functional capacity in $PLWH_{EC}$ [45, 46]. Furthermore, alternative immunological factors have been proposed as contributors to control such as innate effector cell activity, antibody responses, and antibody dependent cell mediated cytotoxicity (ADCC) [31]. The genetic makeup of the host has also been proposed as a contributing factor with human leukocyte antigen (HLA) alleles that can make the host protective (e.g., HLA-B*27 or HLA-B*57) or recessive toward infection (e.g., HLA-B*07 or HLA-B*35) [31, 47, 48]. In general, $PLWH_{EC}$ have strong HIV-1 immune responses together with low level of inflammation [49].

ART initiation in the acute phase has shown the potential to convert the patient into a "post-treatment controller" for up to 10 years [50]. This revelation indicates that there are potential genetic, transcriptomic/proteomic factors, or humoral responses that can mediate a delayed control of viraemia. One study showed how a shorter ART duration in $PLWH_{EC}$ resulted in decreased numbers of HIV-1 infected $CD4^+$ T cells which rebounded after treatment termination [51]. These data therefore imply that the reservoir in some $PLWH_{EC}$ is not always in deep latency, rather, low levels of viral replication are ongoing in these individuals. Thus,

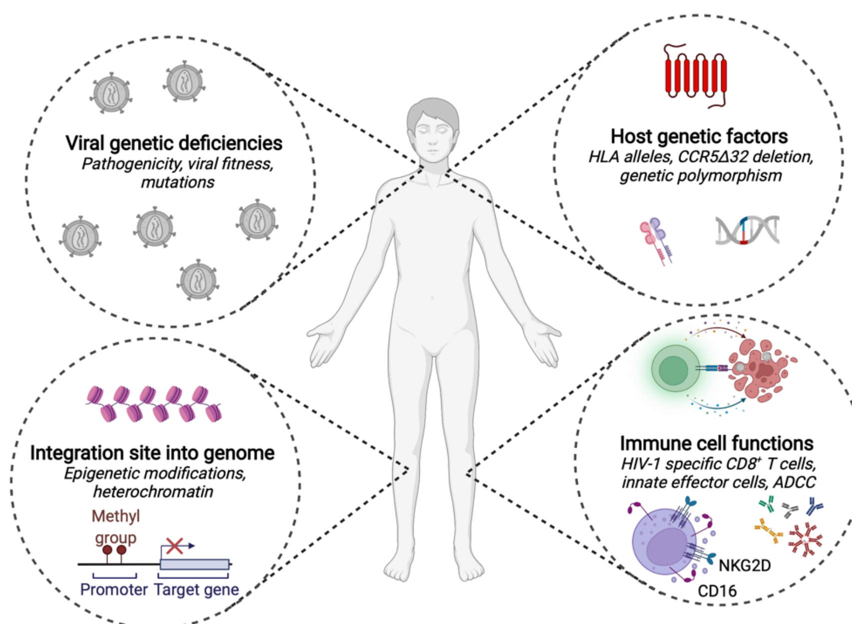

Figure 1: Proposed mechanisms contributing to natural control of HIV-1 infection in $PLWH_{EC}$. Created using Biorender.com.

natural control of HIV-1 infection is heterogenous and most likely mediated through a combination of viral genetic factors, host factors, integration site into the human genome, and the host immunological response [31] (**Figure 1**).

1.1.3 People living with HIV-1 on suppressive ART (PLWH$_{ART}$)

Since its implementation, ART can suppress viral replication to undetectable levels in the blood. These drugs have been developed to target many parts of the viral life cycle and can be classified based on their mode of action, of which the most common are nucleoside or nucleotide reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI), integrase strand transfer inhibitors (INSTI), caspase inhibitors, and entry inhibitors [52]. A complete ART regimen generally consists of a three-drug combination to maximize the efficiency of virus suppression, although efficient dual-drug combinations have been introduced during the last years in clinical care.

Even with a seemingly effective restoration of immune cell functions, PLWH$_{ART}$ have an ongoing inflammatory environment indicated by increased inflammatory markers and dysregulated immune cell functions [53-57]. Additionally, depletion of CD4^{+} T cells in the gut associated lymphoid tissue (GALT) during initial viraemia is only partially restored during ART, leading to translocation of microbial products, e.g., lipopolysaccharide (LPS), that enter the circulation [58-61]. These heightened levels of inflammatory markers together with distorted immune cell function and frequencies contribute to a chronic inflammatory environment in the body.

Immune cell restoration and capacity to decrease inflammatory levels is possibly connected to when individuals started ART. Implementation of ART during seroconversion can reduce the pool of latently infected cells [62, 63]. Restoration of CD4^{+} T cell counts, both in blood and GALT, has also been connected to early ART initiation [60, 64]. Therefore, data implies that ART should be implemented at diagnosis to improve the health and welfare of the patient. This can reduce the inflammatory levels and aid in restoration of the immune system during suppressive therapy in the future. Even so, this does not always apply since a significant proportion of PLWH are diagnosed at a late stage, so called late testers [65, 66]. Earlier guidelines also stated that ART be implemented when CD4^{+} T cells counts dropped below <350 cells/mm^3 [52]. For these individuals, further studies are needed to find alternative strategies that can aid in the restoration of their immune cell functions/frequencies to reduce chronic inflammation and bring the body back to homeostasis.

1.1.4 HIV-1 persistence

The main barrier for HIV-1 cure is the quiescent viral reservoir. It consists of integrated HIV-1, some capable of replication, but to the largest extent transcriptionally defective. The integrated provirus, remaining dormant in the host cell, is generally defined as the latent viral reservoir [67]. HIV-1 latency is a reversible state that occurs after integration of the provirus in the host genome. For not yet known reasons, the transcriptional machinery, specific for the virus, is halted, whereas the normal host transcriptional activity remains intact. The initial pool of latent cells is established early on during HIV-1 infection, is replenished, and persists over time [68-71]. Cessation of ART results in a rebound of the viral load, thus evidence suggests that the reservoir is not affected by ART, rather it only targets transcriptionally

active provirus [72-75]. At a DNA level, the reservoir only exhibits a few nucleotide changes over time on successful ART suggesting a stable HIV-1 reservoir in PLWH [62, 76]. Survival of these cells is possibly dependent on a low level of viral replication together with homeostatic proliferation [77]. Sequence homology in latently infected CD4$^+$ T cells hints toward their clonal expansion, resulting in persistence where the longevity is only dependent on the lifespan of the host cell and its progeny [78-82].

Integrated provirus is characterized based on its transcriptional capacity as inducible and non-inducible, where only 5% of total provirus is considered as replication competent. The remaining 95% is believed to carry deleterious modifications making it incapable of production of infectious virions [83, 84]. Although HIV-1 preferentially integrates into transcriptionally active chromatin regions [85, 86], alternative elements e.g., epigenetic silencing, transcriptional interference, lack of transcription factors, transcriptional repressors, or lack of viral splicing, can contribute to keeping HIV-1 in a latent state [87]. All these aspects contribute to the heterogeneity of HIV-1 latency as integration sites and the suppressive environment play a major role in seeding of the provirus, although only a fraction in transcriptional regulation, as discussed below.

Post-integration, the provirus can be regulated by trans- or cis-acting elements. Trans-acting elements are cellular latency regulating factors such as the chromatin organization, cellular proteins, and signalling ques from the environment [88]. These trans-acting elements are dependent on the availability of transcription factors and accessibility of the promoter, both of which are connected to the activity of the cell. Cis-acting elements refer to structural sequences around the HIV-1 promoter (integration site and chromatin) together with transcriptional interference and viral genetic deficiencies. Genetic deficiencies in the virus can be caused by the error-prone HIV-1 transcriptase, resulting in quasispecies with a reduced viral fitness or immune escape mutations. Alternatively, mutations in the viral genome can be induced directly by the host immune mechanisms, reducing the replication capacity of the virus [89]. To achieve a functional cure for HIV-1, there is a need to understand the underlying mechanisms regulating the quiescent virus and its interaction with the immune system.

1.1.5 Cellular reservoirs of HIV-1

Permissiveness of HIV-1 requires expression of the CD4 surface marker together with a co-receptor for viral entry, most predominantly CCR5 or CXCR4, as mentioned above. Due to its high surface expression, the main reservoir is believed to be CD4$^+$ T cells expressing a memory phenotype while naïve T cells, due to their lower receptor expression, are highly resistant towards infection [90]. Myeloid cells are also a target for HIV-1 due to the expression of CCR5 on the cell surface in monocytes and macrophages, but their contribution to the reservoir is believed to be smaller compared to T cells [91]. Most studies have been performed on peripheral blood, however circulatory CD4$^+$ T cells compromise only 0.25-2% of total lymphocytes, indicating that the majority of these cells are resident in other anatomical compartments. Alternative tissue reservoirs of latent HIV-1 are the lymph nodes,

GALT, other organs, the genital tract, and the central nervous system (CNS) [92, 93] (**Figure 2**). HIV-1 is known to utilize some of the intrinsic properties of these tissues to bolster its persistence. For example, follicular dendritic cells (FDCs) residing within the B follicles of the lymph node can trap viral particles on their cell surface, increasing viral exposure to proximal CD4^{+} T cells [94]. Targeting this reservoir also represents a significant therapeutic challenge due to the low drug penetrative properties of the lymph nodes. In the GALT, HIV-1 can replicate within tissue-resident macrophages. These macrophages are long lived, resilient towards apoptosis during ART, and exhibit low replication, thereby challenging the dogma that macrophages have a short life span, and limited capacity for self-renewal [67, 91]. As all these compartments and cell types have the capacity to carry latent HIV-1, it forms the basis for the need of a multifaceted approach to determine the interplay between all cells and tissues in the human body.

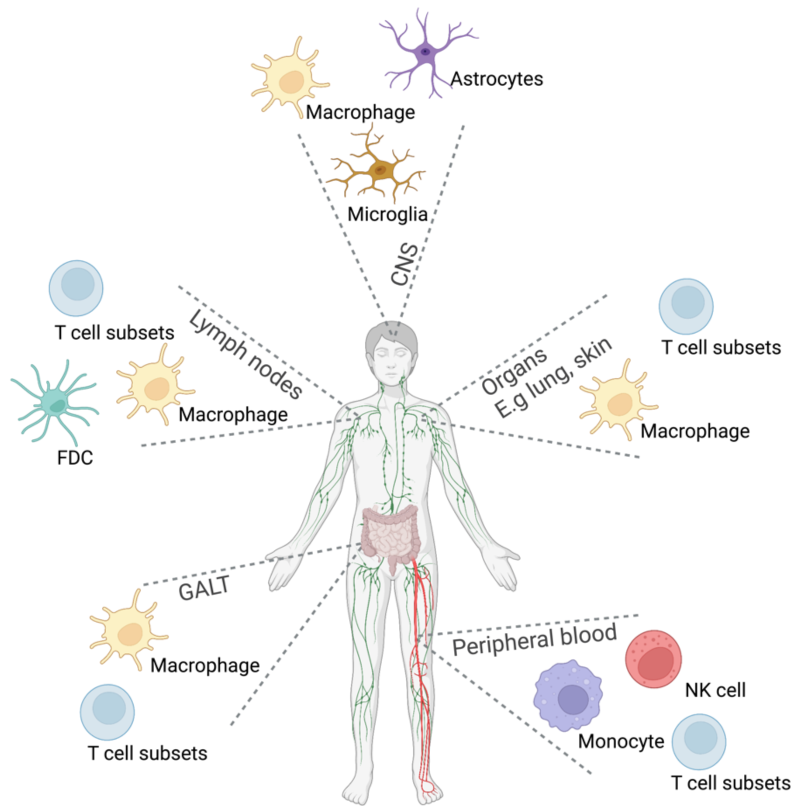

Figure 2: Distribution of the HIV-1 reservoir across cell types and compartments in the body. Created using Biorender.com.

1.1.6 Detection of latently infected cells

HIV-1 particles are approximately 100 nm in diameter, and each consists of a viral envelope that encloses enzymes and two RNA copies of the genome. Once integrated, each host cell is believed to carry one copy of the viral genome, although it cannot be excluded that a limited number of cells carry two copies. Detection of persisting HIV-1 in resting cells is a major hurdle due to the lack of markers associated with the infection. Therefore, current detection methods have focused on measuring DNA (PCR based assays) [95-97], RNA (RNAflow or PCR based assays) [98, 99], protein (detection of viral protein) [100], or the capacity of infected cells to produce virus (viral outgrowth assays (VOA)) [101]. One limitation associated with these techniques, except the VOA, is the lack of identification of replication competent virus. Different detection techniques have also been developed which combine several of these methods to increase the sensitivity and specificity of the detection (e.g., dual RNA and protein detection by RNAscope or FISH-flow or VOA and DNA detection by PCR) [102-104]. In general, the method used for detection of HIV-1 is solely dependent on the application and should therefore be chosen carefully.

1.1.7 HIV-1 cure studies

Over the decades, extensive research has been put into the development of an HIV-1 cure [105]. At the end of the 1990's the Sönnerborg group was involved in one of the first HIV-1 control studies, giving a scheme of four antiretroviral drugs and a therapeutic vaccine to patients with primary HIV-1 infection [106, 107] and we are still pursuing similar studies [108]. The initial discovery of the protective role of the homozygosity of the CCR5Δ32 deletion towards HIV-1 infection [109, 110] has been exemplified by the "Berlin patient" [111] and the "London patient" [112]. In recent advances, a female was transplanted with a haplo-cord transplant of umbilical cord stem cells with the CCR5Δ32 deletion together with a relative's stem cells without the deletion. This has resulted in >14 months remission of HIV-1 in the putatively cured "New York patient" (CROI 2022). Although the technique shows promise it is a complicated, expensive, and dangerous procedure not suitable as a cure for most patients. For all three of these cases, the use of stem cell transplantation was approved as a part of a treatment strategy for secondary leukaemia or lymphomas, and not for HIV-1.

Even as alternative efforts have been put into the development of an HIV-1 vaccine, it won't be applicable to the large population already infected. For these individuals, strategies called "shock and kill" and "block and lock" have been formulated to tackle the global HIV-1 burden. The shock and kill method aims at purging latent HIV-1 from the body through activation with latency reversal agents (LRA), achieving a sterilizing cure [113]. Many of these LRAs are repurposed from the cancer field and act on global transcriptional activation or immunomodulatory effects such as protein kinase c (PKC) agonists, protein kinase B (Akt) activators, or epigenetic modifiers e.g., histone deacetylase inhibitors, to name a few [114]. Although the shock element of this strategy has proven successful in cell culture models, its relevance and capacity to kill has yet not shown sufficient efficacy in clinical trials [115]. Additionally, it is not yet confirmed if the high concentrations needed of the LRAs might induce global toxicities in the body. Immunotherapeutic applications are also being explored in combination with LRAs to accelerate the kill element of the method [116]. The block and lock strategy on the other hand focuses on suppressing HIV-1 transcription and/or reactivation through alternative mechanisms [117]. Theoretically, this method suppresses HIV-1 in a deep latent stage via methods such as epigenetic silencing. This method could potentially achieve a functional cure without ART.

1.1.8 Ageing in HIV-1 infection

Biological ageing is a means to determine the function and activity of the body's inherent responses. It is well known that with age the activity of the immune system decreases together with a decline in cell division and the development of age-associated disorders such as dementia, frailty, cancer, cardiovascular disease etc. Biological age is usually determined by a series of events including telomere shortening, accumulated DNA damage causing genomic instability, epigenetic markers, cellular senescence causing low activity and replication arrest, deregulated homeostasis of proteins (proteostasis), and altered mitochondrial functions resulting in lower energy availability and accumulation of reactive oxygen species (ROS) [118].

Earlier ageing was assumed in PLWH as they were considered to be more frail compared to HIV-1 negative individuals at the same age [119, 120]. These individuals were presumed to age faster and need medical attention at an earlier age, although in most cases this is not the situation. Today, most of the studies available on ageing during HIV-1 are of a pure association design. Some researchers have reported increased markers of epigenetic ageing, both in parts of brain regions and in blood, in $PLWH_{ART}$ [121, 122], while other have shown how ART initiation might reduce epigenetic ageing [123]. Additionally, studies have detected higher incidence of cardiovascular disease [124] and cancer [125, 126], to name a few measured outcomes. In parallel, the PLWH group is often overrepresented with risk factors such as smoking, drug use, coinfections, and/or stress due to other aspects compared to the HIV-1 negative population they are compared to [127]. As all these parameters influence the immune system, and also ageing, it is important to delineate what is an effect of what.

In terms of ageing in PLWH, no consistency exists in the definition. Collectively the terms premature, accelerated, and accentuated ageing, have been proposed. Accelerated and premature ageing crudely describe increased changes over time that arise earlier and increase progressively. Accentuated ageing describes an increased burden of age-related damage that occur at the same age as the general population and is static over time [128]. Still today, the relationship between earlier ageing and HIV-1 infection is not clear. To be able to appropriately study and assess this area, the definition for ageing in PLWH needs to be clarified in terms of 1) what it refers to; 2) what the presumed consequences are; 3) what causes it; 4) what is it we compare it to; and, importantly, 5) how is ageing accurately quantified. Additionally, as ART has a systemic effect in the body it could influence ageing in this population. To eliminate this confounder, future studies on people using PrEP for prolonged periods of their life could possibly help to unravel the effect of ART itself as a contributor to the ageing process. However, it is very important to be careful in interpretation of results, including the clinical relevance, and the choice of wording when discussing ageing in HIV-1 infected patients since it significantly contributes to stigmatization, importantly the self-stigmatization, of individuals. Still, it is important to understand the biological ageing process beyond association studies, and its impact on the immune system and wellbeing of PLWH, so that an intervention strategy can be developed for healthy ageing.

1.2 THE IMMUNE SYSTEM

The immune system is one of our main barriers against foreign pathogens. It mediates recognition and clearance of infecting microbes as well as infected cells through unspecific, short lasting innate immunity and specific, long-lived, adaptive immune responses. To counteract infections, immune cells have antiviral defence mechanisms that sense viral products by pattern recognition receptors (PRRs) such as cyclic GMP-AMP synthase (cGAS), interferon inducible protein 16 (IFI16), and toll like receptor 8 (TLR8) [129]. This activates signalling pathways specialized in mediating an effective antiviral response to clear the pathogen e.g., interferon (IFN) signalling, inflammasome signalling, activation of restriction factors, and regulation of adaptive immune responses against the virus. Before the adaptive immune response kicks in, innate immunity can control early stages of HIV-1 infection [130]. HIV-1 recognition by PRRs leads to the production of innate immune cells and the release of soluble factors e.g., proinflammatory cytokines, chemokines, and IFNs. However, HIV-1 has also evolved mechanisms to counteract these responses as HIV-1 accessory proteins can suppress some of these antiviral defence mechanisms for its survival and spread [129]. For example, they can suppress antiviral IFN stimulatory genes by impeding components of IFN and NFκB signalling. They can also induce degradation of IFN regulatory transcription factors by lysosomal or ubiquitin mediated pathways. Additionally, vif can counteract host restriction factors such as apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G) [129, 131].

HIV-1 is known to deplete immune cells through the viral pathogenic cycle, yet infection does not correlate with paresis of its activity. On the contrary, a hallmark of HIV-1 infection is a chronic immune activation where cells have a rapid turnover and are susceptible to activation-induced apoptosis to restrain spread of the virus [132]. Therefore, while the immune system represents a sophisticated network of anti-pathogenic effector cells and molecules aimed at clearing infected cells and returning to homeostasis, HIV-1 can modulate immunological functions to promote its survival and spread.

1.2.1 Myeloid cell lineages

Cells of myeloid lineages like unspecific monocytes, macrophages, and dendritic cells (DC), together with myeloid derived suppressor cells (MDSCs), serve as the first line of defence against infection (**Figure 3**). Of these, the first three belong to the mononuclear phagocytes (MNPs) that are specialized in phagocytosis and antigen presentation and are crucial mediators of both innate and adaptive immune responses. In humans, monocytes mainly play a role in host immune surveillance and replenish the macrophage and DC pools upon differentiation [133]. Monocytes are generally divided based on their surface expression into classical (CM: $CD14^{+}CD16^{-}$), intermediate (IM: $CD14^{+}CD16^{+}$), and non-classical (NCM: $CD14^{-}CD16^{+}$), where CD16 is a surface marker that shows a phenotype characteristic of terminal activation and differentiation. $CD16^{+}$ monocytes generally have a higher expression of CCR5 and are therefore more susceptible to HIV-1 infection [134]. Activation of MNPs results in the production of pro- and anti-inflammatory molecules (soluble CD14 (sCD14), IL-6, soluble CD163 (sCD163), tumour necrosis factor (TNF), and interleukin-1 (IL-1)) to recruit adaptive immune cells and resolve inflammation [135, 136]. These molecules also have an array of additional functions that can have antiviral properties to reduce viral fitness and help counteract infection directly and indirectly [10, 137, 138]. The dysregulation and

activation of myeloid cell lineages is largely reduced during ART initiation, but the effect is not completely ameliorated.

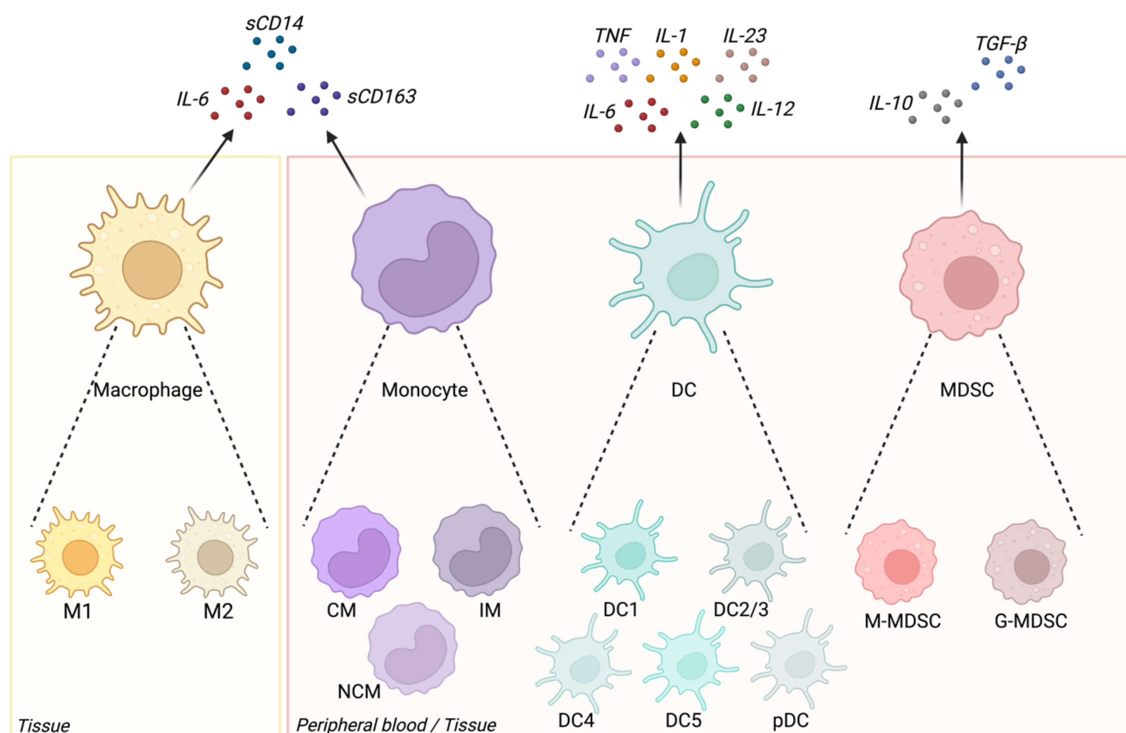

Figure 3: Mononuclear phagocytes (MNPs) (macrophages, monocytes, and dendritic cells (DCs)) and myeloid derived suppressor cells (MDSCs) and their respective subpopulations used in this thesis. Created using Biorender.com.

Myeloid cells have several host cell restriction factors to minimize establishment of HIV-1 infection, although tissue resident myeloid cells comprise a fraction of the HIV-1 reservoir [91]. These cellular restriction factors are active during the early stage of HIV-1 replication and include APOBEC3G and sterile alpha motif domain and HD domain-containing protein 1 (SAMHD1). APOBEC3G is a cytidine deaminase that induces G to A hyper-mutation in the viral DNA which disrupts uncoating and reverse transcription of the virus, while SAMHD1 decreases the deoxynucleoside triphosphate (dNTP) pool so that the virus has lower capacity for transcription [93, 139]. If infection is established in myeloid cells, their phagocytic capacity can be directly or indirectly dysregulated [140-142]. DCs can also trap viral particles on the cell surface, mediating spread to target cells in tissues and lymph nodes. Due to their capacity to migrate across the blood-brain barrier, myeloid cells are also believed to contribute to infection of the CNS and as a consequence neurocognitive impairment during HIV-1 infection in the majority of untreated patients [133], although ART reduces this problem to a low level. Therefore, even as the function of myeloid cells is mostly restored during ART, these cells can still elicit a global spread by infecting bystander cells while contributing to chronic inflammation.

1.2.2 T lymphocytes

T lymphocytes are further divided based on their cell surface markers and effector characteristics into $CD4^+$ or $CD8^+$. The $CD4^+$ lymphocyte population provides essential T

cell signalling to their CD8⁺ counterparts and comprises a variety of subsets with specific characteristics and cell surface markers (e.g., T helper (Th) 17 (Th17,) regulatory T cells (Tregs), Th1, etc). The CD8⁺ lymphocyte population consists of cytotoxic T cells that mediate the lysis of infected cells, as well as suppressor cells that restrain immunological responses. Naïve lymphocytes are developed in the lymph nodes where initial antigen encounter results in a selective propagation of cells carrying the recognition capacity for the new pathogen. Activation of T cells occurs through stimulation of the T cell receptor (TCR) which results in the upregulation of activation markers such as CD38, CD69, and HLA-DR, and differentiation into effector or memory T cells. Infection can also induce the propagation of HIV-1 specific cytotoxic T lymphocytes that can mediate the clearance of infected CD4⁺ T cells, resulting in their depletion (**Figure 4A**) [143, 144].

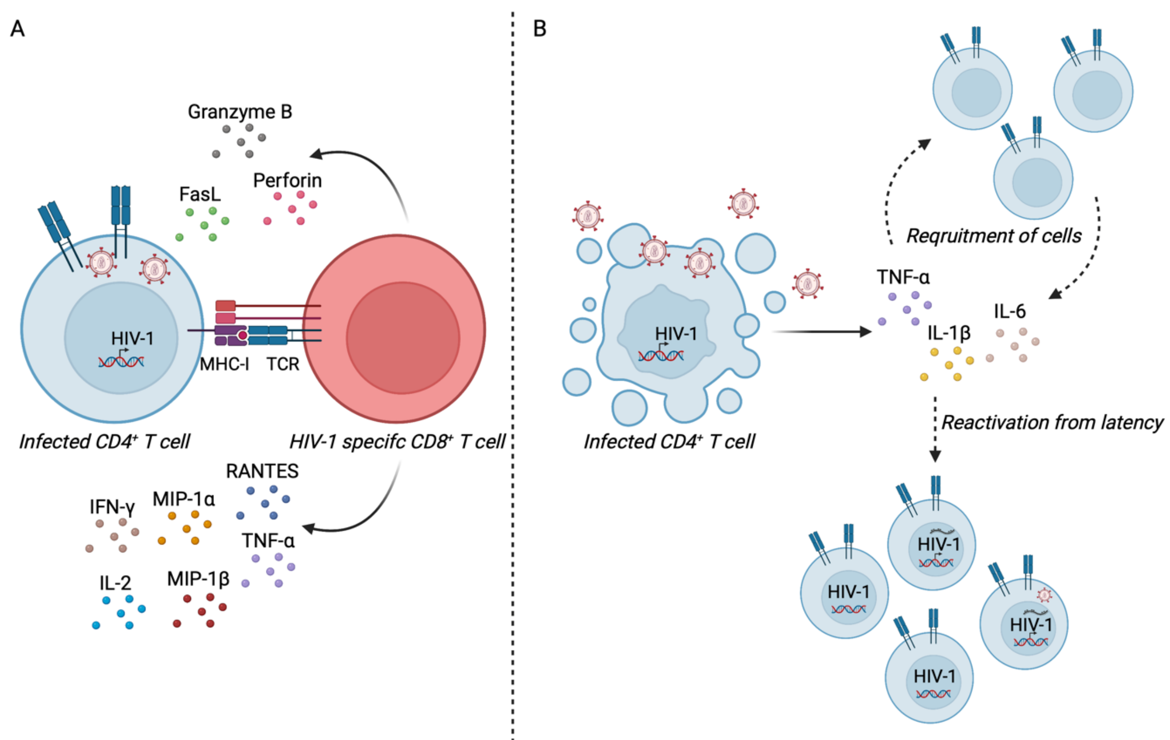

Figure 4: Lymphocytes in HIV-1 infection. (A) Cell mediated killing of infected CD4⁺ T cell by HIV-1 specific CD8⁺ T cell. (B) Result of cell death of HIV-1 infected CD4⁺ T cell mediating recruitment of new target cells to the site and reactivation from latency of bystander cells. Created using Biorender.com.

The longevity and stability of the viral T cell reservoir is driven by low ongoing viral replication, sanctuaries with low drug penetration, homeostatic proliferation, and cell-cell transmission [90, 145]. During HIV-1, CD4⁺ T cells are depleted, as they are the main targets for HIV-1, whereas of CD8⁺ T cell activation is associated with HIV-1 progression. This chronic activation leads to induced death of T cells releasing apoptotic microparticles that can mediate death in bystander cells. From here a vicious pathogenic cycle is created from infected cells, sustaining the latent reservoir. This is caused by dying CD4⁺ T cells releasing pro-inflammatory cytokines, e.g., IL-1β, IL-6, TNFα, promoting virus production in latent cells and recruitment of uninfected cells to the site (**Figure 4B**) [132]. The provirus itself, although generally transcriptionally silent, can exhibit a low ongoing gag production in the absence of reactivation [146]. This continuous low level of viral replication contributes to an ongoing immune triggering and inflammation that can lead to exhaustion, as discussed below.

As a result of immune dysfunction, in the form of deregulated B cells and T cell exhaustion, clearance of latently infected cells is profoundly inefficient.

1.2.3 Chronic inflammation during suppressive HIV-1 infection

During ART, there is a convergence between HIV-1 persistence and residual immune activation [147]. Even as therapy is initiated, suppressive ART does not fully restore immune cell functions and the residual viral replication contributes to a chronic inflammatory environment [148, 149]. This persistent immune activation, in combination with coinfections, such as cytomegalovirus (CMV), can have severe consequences by contributing to the development of non-AIDS related comorbidities such as cardiovascular disease, cancer, frailty, neurological complications, and liver or kidney disease [127].

Chronic inflammation can partially be defined by continuously high levels of specific pro-inflammatory molecules in the circulation, e.g., IL-6 and c-reactive protein (CRP), markers of coagulopathy (D-dimer and fibrinogen), and sCD14 [127]. The driving force behind it is a combination of: 1) low levels of viral replication that activates innate and adaptive immune responses; 2) pyroptosis of infected cells releasing inflammatory molecules; 3) coinfections such as CMV, other herpesvirus, or hepatitis C virus (HCV); and 4) CD4^{+} T cell decline that can promote homeostatic proliferation and generation of effector T cells furthering a pro-inflammatory environment in the body [150]. Additionally, microbial translocation in the GALT can cause LPS to bind soluble or anchored CD14 on monocytes leading to the release of pro-inflammatory cytokines [127], as well as translocation of flagellin [151]. This relentless triggering of immunological activities never allows the body to return to homeostasis, irrespective of treatment status. Prolonged chronic activation of the immune system can also induce an irresponsive, irreversible state in cells defined as cellular senescence. This is a phenomenon resulting in a decrease in cell populations, functions, proliferative capacity, and increase in the number of terminally differentiated T cells [152].

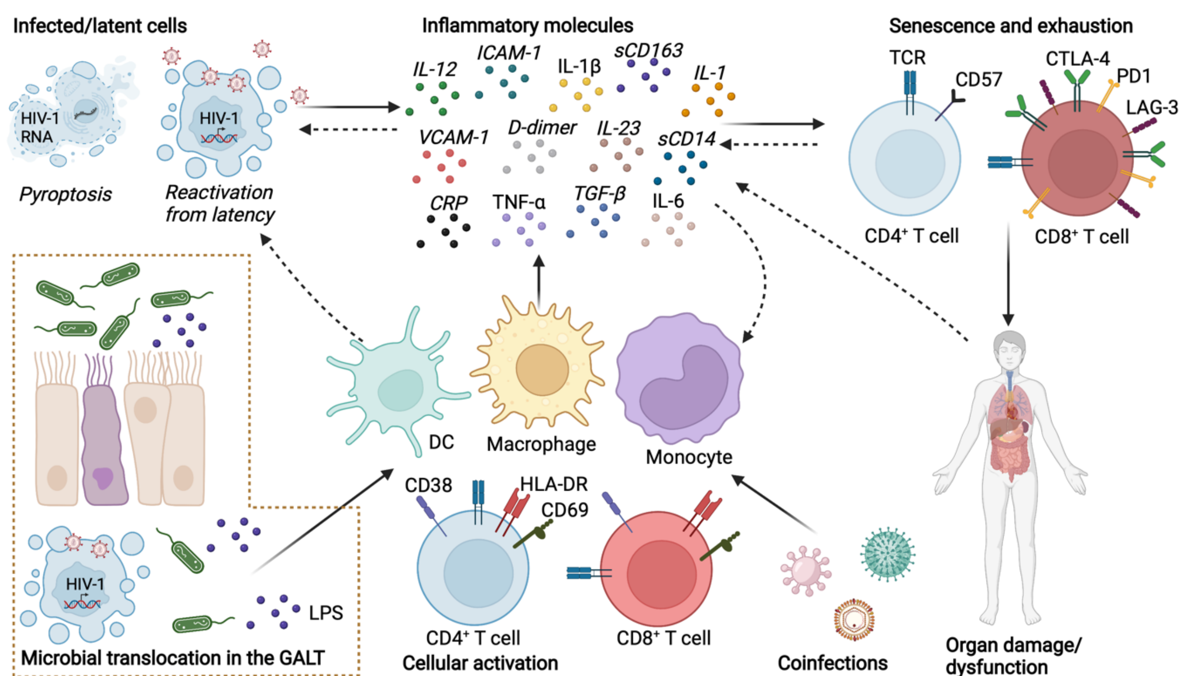

Figure 5: Proposed mediators of chronic inflammation during suppressive therapy in PLWH_{ART}. Created using Biorender.com.

Chronically activated T cells may develop an exhausted phenotype. Immune cell exhaustion is associated with an attenuation of cellular and effector functions due to increased expression of inhibitory receptors on the cell surface [153]. T cell exhaustion seems to be highly dependent on glycolysis and mitochondrial mass and functions [154, 155]. Loss of effector functions includes reduced production of IL-2, TNF, and other cytokines which play an important role in the pro-inflammatory response. In addition, decreased expression of immune checkpoint markers e.g., killer cell lectin-like receptor subfamily G member 1 (KLRG1), coupled with upregulation of inhibitory receptors such as programmed death-1 (PD1), cytotoxic T lymphocyte antigen-4 (CTLA-4) and lymphocyte activation gene 3 protein (LAG-3) also contributes to dysfunctional T cell responses [153, 156]. Exhausted cells are more susceptible to anergy and deletion, resulting in alterations to the overall T cell composition, organ damage, or dysfunction, and contribute to several comorbidities. Many strategies have been implemented to reduce chronic inflammation in PLWH using anti-inflammatory agents, treatment of coinfections, reduction of microbial translocation, and improving immune recovery [150]. Still, chronic inflammation remains a serious condition that can diminish the body's capacity to return to homeostasis and possibly contribute to earlier onset of age-related diseases (**Figure 5**).

1.3 IMMUNOMETABOLISM

While metabolism describes how the cell sustains its energetic demand, immunometabolism defines the interplay between metabolism and immune cell functions. The metabolic state of a cell is one of the determinant factors specifically regulating activity of different cellular phenotypes upon stimulation. Metabolism is altered to sustain the need for increased biomolecule or energy production that results in alterations of immune function such as activation stage, effector function, and overall status of immune cells. Therefore, there is a need to understand the link between metabolic processes and immune function as it is one of the key drivers in regulating immune cell activity in disease states. In the present thesis, the term "metabolic reprogramming" is used to describe alterations in metabolic signalling and consequently immune cell functions. During controlled HIV-1 infection, alterations of these pathways can partly be a consequence of the persisting virus but also the chronic inflammatory environment causing secondary alterations to immune cell subsets.

1.3.1 Metabolism of cells

Metabolism is defined by both the generation of ATP, to sustain the cell with energy and amino acids (catabolic), and the production of biomolecules needed for synthesis of nucleic acids, lipids, and proteins (anabolic) [157]. In the context of energy, metabolic adaptation can be aerobic or anaerobic depending on oxygen availability that ranges from 1-5% in body compartments and up to 12% in arterial blood [158, 159]. General aerobic metabolism uses the three pillars of central carbon metabolism (CCM), namely 1) glycolysis for glucose to acetyl coenzyme A (acetyl-CoA) conversion that fuels both the 2) tricarboxylic acid (TCA) cycle and 3) the pentose phosphate pathway (PPP) (**Figure 6**). Additionally, the TCA cycle is coupled to oxidative phosphorylation (OXPHOS). The process fuelling the TCA cycle and

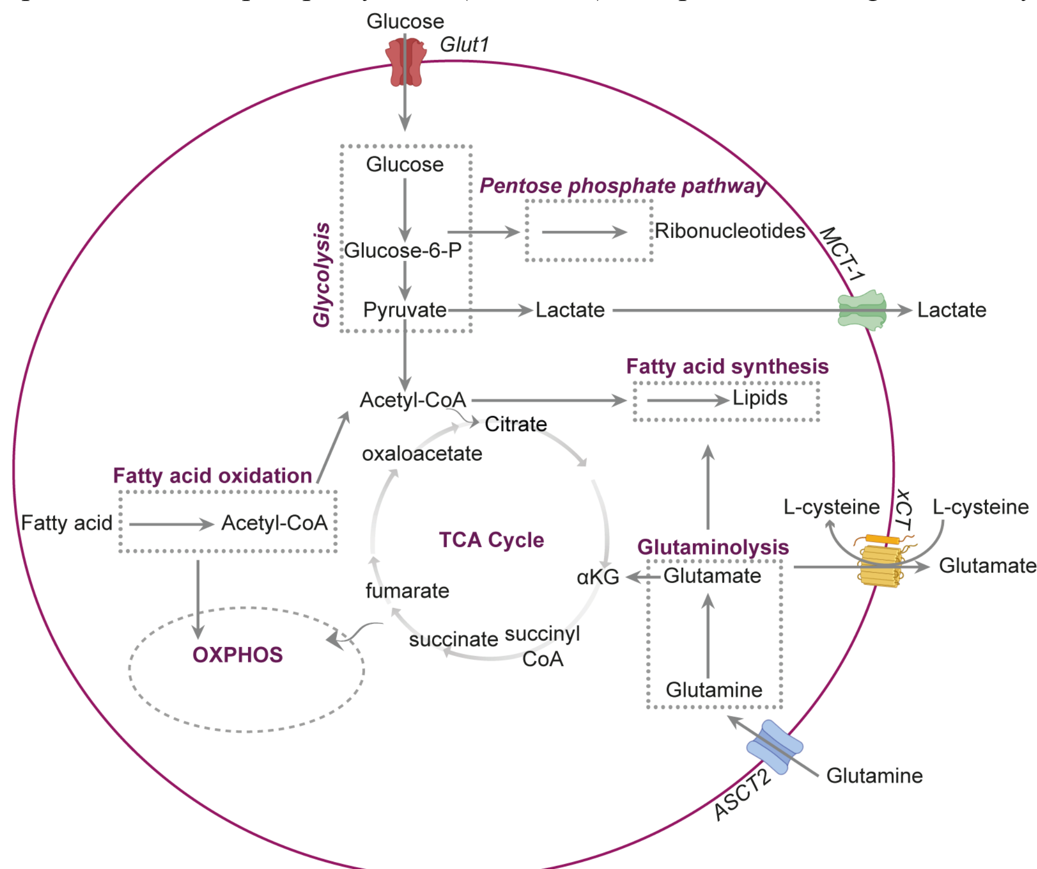

Figure 6: Cellular metabolic pathways. Adapted from **paper V**.

OXPHOS is relatively slow but generates a high amount of ATP. Anaerobic metabolism, on the other hand, is a faster but less energy efficient process that utilizes glycolysis to create pyruvate which feeds lactate production [160].

Within the immune system, naïve and memory T cells have an inherent metabolic signature of low energy production [161]. Therefore, they use catabolic processes to sustain basal cellular processes and chemotaxis through oxidation in the mitochondria. Activation of a T cell results in a higher demand of energy and biomolecule output, thereby shifting the metabolism to an aerobic glycolysis, independent of oxygen availability [162, 163]. This metabolic shift called the Warburg effect, well characterized in cancer cells, is an essential adaptation to withstand the high metabolic rate needed for proliferating cells which favour anabolism and biomass production [160, 164]. Although termed as a shift in metabolism it is a phrase describing the preferential pathway used. This means that upregulation of one pathway does not denote a shutdown of the other pathway, it is just used at a lower level. During T cell activation, this means an upregulation of glucose transporters (e.g., Glut1, Glut3) to provide energy for sufficient effector functions by aerobic glycolysis, while utilizing lower levels of TCA cycle and OXPHOS [165, 166]. Upon preferential pyruvate fermentation into lactate, glutaminolysis can also act as a complement for the TCA cycle. Glutaminolysis is the process of glutamine conversion to alpha-ketoglutarate (αKG) which feeds into the TCA cycle [160]. This can be viewed as a bypass system to maintain energy and biomolecule production through the lower parts of the TCA cycle and OXPHOS.

Activation of monocytes, like lymphocytes, results in a suppression of OXPHOS while upregulating glycolysis in response to pro-inflammatory stimuli [167, 168]. Therefore, the Warburg effect is not a phenomenon specifically for cancer cells, as it is also observed during normal cellular activation for rapid clonal expansion. In terms of biomolecule production, intermediates for lipid, nucleotide, and amino acids (AA) for macromolecule synthesis are created through the metabolic processes. This is achievable by fatty acid oxidation (FAO), generating acetyl-CoA and NADH for energy production and fatty acid synthesis (FAS) manufacturing molecules needed in the cell [160]. In parallel to glycolysis, the PPP is an anabolic process utilizing glucose for generation of NADPH, precursors for nucleotide synthesis, and maintaining redox homeostasis [169].

Redox homeostasis is like a scale weighing the production of and elimination of ROS to avoid development of oxidative stress. ROS (e.g., hydrogen peroxide (H_2O_2) and free radicals such as superoxide anion (O_2^-)) are an umbrella term for different molecular oxygen derivates that can be produced from cellular enzymatic reactions such as metabolic pathways. A large amount of ROS is produced when generating energy in OXPHOS [170], while transition into aerobic glycolysis reduces ROS production in cells [171]. Within immune cells, the generation of ROS is needed for appropriate signal transduction, but if levels are above homeostasis, the results can be detrimental. During normal conditions, redox homeostasis can be maintained by ROS neutralization through enzymatic reactions revolving around antioxidant defence mechanisms [170]. To resolve ROS, the NADPH produced from PPP is involved in redox homeostasis by converting oxidized glutathione (GSSG) to reduced glutathione (GSH). Additionally, PPP produces ROS for sequential signalling transduction. Simultaneously, metabolites like pyruvate can act as scavengers and aid cells against ROS

induced damage [172, 173], while glutamine is important in GSH antioxidant defence to maintain low inflammatory levels in the body [174]. The metabolic status of immune cells is therefore a tightly regulated network determined by the activation status and effector functions of a cell, as well as adaptation to the environment.

1.3.2 Pathways regulating metabolism

Metabolic regulation occurs through multifactorial external stimuli including glucose and glutamine availability, cellular activation through TCR stimulation, growth factors, cytokines, and oxygen levels. Therefore, a plethora of signalling pathways can regulate the metabolic adaptation based on external stimuli. Some of these pathways, crucial for cells to meet their energetic need, are phosphoinositide 3-kinase (PI3K), Akt, mammalian target of rapamycin (mTOR), hypoxia inducible factor (HIF), AMP-activated protein kinase (AMPK), and cellular myelocytomatosis oncogene (c-Myc) [160]. In a low oxygen setting, the mTOR/HIF signalling pathway is activated, shifting from OXPHOS to glycolysis [175]. mTOR is a serine/threonine protein kinase divided into two complexes, mTORC1 and mTORC2 generally believed to increase glucose uptake through Glut1 upon activation [176]. Additional research has shown how mTORC1 contributes to metabolic regulation through lipid metabolism, mitochondrial biosynthesis, and induction of HIF signalling, while mTORC2 plays an important role in glucose metabolism such as glucose uptake, glycolysis, gluconeogenesis as well as OXPHOS [177, 178].

HIF signalling plays a major role in adaptive responses to cellular stress, and it is a master regulator of transcriptional activity of more than 100 downstream genes involved in cell survival, proliferation, differentiation, angiogenesis, and apoptosis [175]. HIF exists in two isoforms, the constitutively expressed oxygen sensitive HIF-1α and oxygen insensitive HIF-1β (alternatively named ARNT) that upon activation, dimerize and translocate into the nuclei for transcriptional activation of target genes. In metabolism, HIF signalling is the major mediator of glycolysis. It also contributes to reduced TCA metabolites through lowered oxidation of αKG to succinate, leading to increased citrate production that can be utilized for fatty acid metabolism [179].

In the presence of oxygen, there are several pathways that can regulate metabolism based on external stimuli and demand. To promote anabolic metabolism PI3K/Akt/mTOR/HIF signalling is upregulated. On the other hand, when ATP levels are low AMPK signalling is induced to promote catabolic processes [180]. Furthermore, c-Myc activation is essential in the activation-induced metabolic reprogramming of T cells [181]. Together, these pathways organize a complex network of regulatory elements for cells to retain specific cellular functions during both normal homeostasis, infection, and disease.

1.3.3 Immunometabolic reprogramming during HIV-1 infection

Metabolic reprogramming of immune cells is a crucial factor regulating activation and adaptation to the environment, external and internal stimuli, as discussed above. At the same time, viruses have developed strategies to use metabolic pathways and exploit the host metabolic machinery for their own replication and spread [182]. During HIV-1 infection, the metabolic activity of a cell plays a pivotal role in susceptibility to infection. $CD4^+$ T cells with elevated glycolysis and OXPHOS, sustained by increased glutamine uptake, are more

permissive to HIV-1 [183-185]. This correlates to some extent with the profile of activated cells, as described above, whereas naïve and resting T cells are more resistant towards HIV-1 infection [186, 187]. Simultaneously, high glucose availability itself is a factor increasing susceptibility and transcription of HIV-1. This is caused by a ROS-mediated upregulation of HIF-1α and CXCR4 and uptake of glucose through Glut1 [188, 189]. The same importance is displayed by metabolic adaptation after HIV-1 have infected a cell. Virus propagation is an energy demanding process that requires both synthesis of macromolecules and hijacking the cellular biosynthesis machinery [190]. To meet the energy requirements, infections such as HIV-1 induce protein levels of glucose receptors, elevating glycolysis to support virus propagation in both monocytic and lymphocytic cell subsets [191-195]. To promote glycolysis, HIV-1 can also regulate the activity of glycolytic enzymes to its advantage [196]. While upregulated in PLWH, these glycolytic enzymes can have dual activity as some have described antiviral properties [197, 198]. Inhibition of glycolysis also induces higher cell death in infected compared to uninfected cells [184]. Thus, the virus forces a higher metabolic activity in infected cells to meet its energy demands by promoting aerobic glycolysis for energy and biomolecule synthesis.

The mTOR pathway is important during metabolic reprogramming, as discussed above, while also a crucial regulator of HIV-1 latency and memory T cell functions [199-201]. In $PLWH_{EC}$, the capacity of HIV-1 specific $CD8^+$ T cells to suppress infection is dependent on glucose metabolism and OXPHOS controlled by the mTORC2 pathway. On the other hand, $PLWH_{ART}$ rely to a higher extent on glucose metabolism regulated through mTORC1 [202]. Therefore, the upregulation of mTORC2 in $PLWH_{EC}$ strengthens the evidence of a regulatory role in HIV-1 persistence [203]. Prior to loss of elite control status, individuals display a metabolic shift to aerobic glycolysis together with deregulated mitochondrial activity, immune activation, and oxidative stress [204]. Still, the question remains if it is production of virus that induces the metabolic shift or if it is alternative mechanisms, inducing upregulation of glycolysis, that contribute to loss of viral control.

Increased basal markers for aerobic glycolysis and high surface expression of Glut1 have been detected in $CD4^+$ and $CD8^+$ T cells from $PLWH_{ART}$ [183, 194, 205]. Glut1 expression on $CD4^+$ T cells, predominantly displaying a central or naïve phenotype, correlates to the immune activation marker HLA-DR, while glycolytic metabolism correlates to mitochondrial density [194, 205]. Therefore, it can be postulated that these cells have an enriched mitochondrial capacity to rapidly respond upon infection. Some have proposed that Glut1 is a marker of activation of T cells [194]. On the other hand, it has also been shown that Glut1 expression is not associated with activation markers on $CD8^+$ T cells [206]. It is probable that Glut1 does correlate with T cells activation, although it is unlikely to be an independent marker alone. Additionally, monocytes also express higher levels of Glut1 in $PLWH_{ART}$ [193]. The memory capacity of monocytes, otherwise specific to adaptive immune responses, is highly dependent on glycolysis and controlled by the Akt/mTOR/HIF pathway [168, 207].

Plasma metabolomics in PLWH have shown how HIV-1 infection is associated with mitochondrial dysfunction and a decrease in fatty acid oxidation in the mitochondria (β oxidation) while oxidation in the smooth endoplasmic reticulum (ER) is enriched (Ω-

oxidation) [208]. Additionally, in infected macrophages, vpr protein levels can contribute to dysregulation of mitochondrial glutamate metabolism [209]. When cells utilize glutaminolysis, cell growth is promoted through mTORC1 activation, simultaneously inhibiting autophagy [210]. Consequently, augmented mitochondrial activity could contribute to deregulated immune cell functions. Moreover, the monocytic reservoir is sustained by increased glycolytic pathways for PPP and pyruvate metabolism while downregulating key mitochondrial enzymes [211]. It has also been shown that latently infected macrophages mainly use glutaminolysis, together with glucose and fatty acid metabolism, indicating a metabolic shift from OXPHOS upon entering quiescence [212]. While glutamine levels are important for production of GSH to maintain redox homeostasis [174], extracellular glutamate can be involved in neurological disorders during HIV-1 infection [213]. To resolve any damage both lactate and pyruvate can act as scavengers to reduce glutamate mediated neurotoxicity [214, 215]. In the context of HIV-1, this implies that a metabolic shift can contribute to chronic inflammation during suppressive ART.

Besides being important for activation of cells, glycolysis is needed for T cells to exert their effector functions, such as IFN-γ production [216] and TNF secretion from monocytes [217]. In the latter study, the authors also showed how lactate can suppress the secretion of TNF, thereby reducing their proinflammatory phenotype. High lactate levels can also supress HIV-1 specific $CD8^+$ T cell functions [218] while αKG can be involved in modulation of the epigenetic landscape [219]. This shows how individual metabolites also play a role in the regulation of immunometabolic processes. Although not completely elucidated, the dynamics of metabolite concentrations from cellular metabolism can both work in favour and against immune cell functions (**Figure 7**). To what extent this reprogramming occurs due to infection or as a consequence of alternative comorbidities remains to be elucidated.

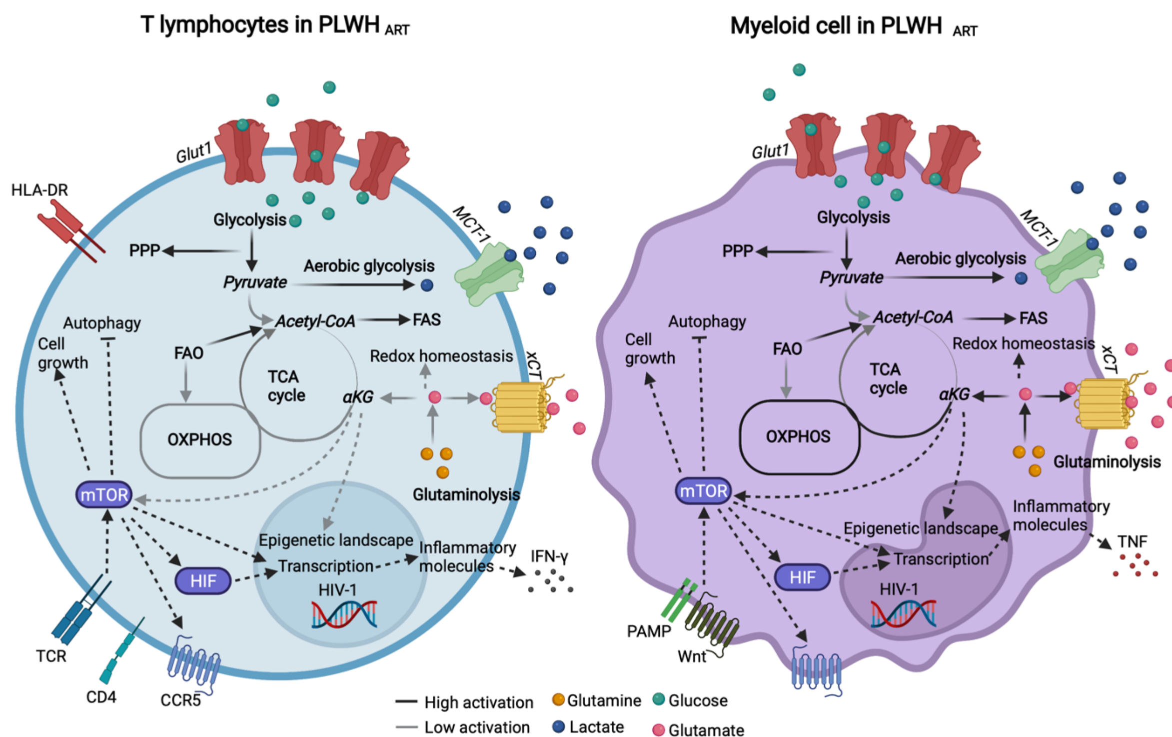

Figure 7: Hypothetical immunometabolic reprogramming in T lymphocytes and myeloid cells in PLWH$_{ART}$ based on current literature Created using Biorender.com.

In conclusion, several studies have been performed to understand how immunometabolism might play a role during infection, yet the plethora of metabolic changes induced during suppressive HIV-1 and its effect on immune cell functions is not yet fully elucidated.

1.4 OMICS DRIVEN DATA ANALYSIS

Technological advancements have rapidly developed the field of high throughput data analysis, allowing a global snapshot of different levels of biological processes from the genome to the metabolome. On a single omics level, these unbiased data driven scientific analyses have shown huge success in understanding physiological aberrations during disease conditions [220]. Today, there are several useful initiatives and tools available online where different layers of omics data, or interactions of omics layers, have been catalogued e.g., Kyoto encyclopaedia of genes and genomes (KEGG) [221], Human Protein Atlas [222], and Human Metabolic Atlas [223].

Multi-omics analysis is the combination of single omics data levels such as genomics, transcriptomics, epigenome, proteomics, metabolomics, or the microbiome, all of which can provide useful information about biological processes and functions [224]. Through this combination, multi-omics analysis can underpin important information during disease states. The flow of biological information generally follows a top-down approach where the genome is translated into mRNA, protein to metabolites, and finally a response will be seen in a phenotype. Alternatively, as the information will decrease per step in this line, a bottom-up approach can be used. In a bottom-up approach, even as a large fraction of information will be lost along the way, this phenotype first driven approach can target the analysis from a higher level to find in-depth differences. Integration of data from several omics layers is a strategy in the system biology approach [225]. This is a newer field where biological processes are aimed to be understood by combining several layers of information while also integrating them into pathways and connecting networks. In HIV-1 infection, both single level and integrative-omics analysis have greatly contributed to our understanding of physiological modulations and phenotypic aberrations during the disease state [226-228]. Also, a seminal study used multi-omics analysis to understand immunometabolic regulation during HIV-1 infection of $CD4^+$ T cells [229]. By combining these single omics levels, the authors could stratify the data on a more complex level.

As with all scientific analyses, the risk of bias can be high. Introduction of bias can be minimized by using a well-defined cohort, thereby eliminating the rejection of a null hypothesis based on confounding effects. It is also imperative to have appropriate statistical and bioinformatics tools available so that an accurate interpretation of the data can be performed. Although the omics data used in the separate manuscripts of this thesis are generally restricted to one level, the combined effort of our group is to provide a multi-omics level system biology approach to answer complex biological questions.

2 RESEARCH AIMS

The overall aim of the project was to investigate the effects of HIV-1 persistence during elite control and long-term successful antiretroviral therapy. Furthermore, we aimed to understand how HIV-1 persistence is influenced by the immunometabolic profile of immune cell subsets.

The specific aim for each paper was:
Paper I: $PLWH_{EC}$ is a heterogeneous group that is characterized by the individual's ability to naturally control HIV-1 infection in the absence of ART. However, the exact mechanisms underlying the $PLWH_{EC}$ phenotype are to a large extent unknown. By integrating proteomic and transcriptomic data, we aimed to investigate features distinct to the $PLWH_{EC}$ phenotype among males to identify signalling pathways which may be involved in their natural viral suppression.

Paper II: Controlled HIV-1 infection, either natural ($PLWH_{EC}$) or ART induced ($PLWH_{ART}$), still results in a modulation of the immune system. In this paper we aimed to compare the immune cell phenotype between $PLWH_{EC}$ and $PLWH_{ART}$ in relation to HIV-1 persistence.

Paper III: Long-term suppressive ART does not normalize the frequency and activity of immune cells to the same level as in HIV-1 uninfected individuals, or individuals with natural control of HIV-1. Therefore, we sought to understand and infer changes in the immune system in $PLWH_{ART}$ compared to $PLWH_{EC}$ and healthy controls on a multisystem level. Using multidimensional omics data and pharmacological modulation we studied how such changes could affect HIV-1 reservoir dynamics and the immune senescence profile.

Paper IV: HIV-1 chronicity can influence long-lasting metabolic flexibility and adaptations during suppressive ART. In this paper, we conducted plasma metabolomic analysis to understand the metabolic modulations during suppressive ART in the Swedish InfCareHIV cohort. Herein, we aimed to characterize the cell populations that mainly contributed to changes in the metabolic environment during long-term suppressive therapy.

Paper V: Like paper IV, we wanted to evaluate if the modulated metabolic environment was consistent on a global scale in two cohorts from low- and middle-income countries (namely, Cameroon and India). We employed plasma metabolomic analysis and *in vitro* experimental models to understand metabolic reprogramming during long-term ART and its potential role in ageing of PLWH.

3 METHODOLOGICAL CONSIDERATIONS

In the prospective papers included in the thesis (see Appendix), all experimental methods are described in detail. This section is allocated for motivating and describing the specific methods and reagents used.

3.1 ETHICAL CONSIDERATIONS

All papers included in this thesis used donor and patient samples. Ethical permits were granted prior to commencement of the projects. **Papers I-IV** were conducted on the Swedish InfCareHIV cohort. This nationwide observational cohort was established by Prof. Anders Sönnerborg in 2004, and by 2008 all HIV-1 clinics in Sweden had joined. Presently, the InfCareHIV cohort includes >99% of all diagnosed PLWH in Sweden. The cohort also covers a substantial proportion of PLWH dating back to the 1980s from the Karolinska University Hospital, South Hospital in Stockholm, and Sahlgrenska University Hospital in Gothenburg. **Paper V** was conducted on two separate cohorts from Cameroon and India. All studies were performed in accordance with the Declaration of Helsinki and informed consent was received from all participants. All samples were delinked before analysis in respective paper. For **papers I-IV** ethical approvals were given by the Regional Ethics Review Board of Stockholm. For **paper V**, ethical approvals were obtained from the Cameroon National Ethics Committee for Human Research, the Institutional Ethics Committee of the National Institute for Research in Tuberculosis India, and the Institutional Review Board of the Government Hospital for Thoracic Medicine India. Also, the application was waived by the Regional Ethics Review Board of Stockholm. Specific information on selection criteria, definition, and characteristics are listed in each respective paper.

3.2 SAMPLE COLLECTION, ISOLATION, AND PROCESSING

For samples included from the InfCareHIV cohort, whole blood was collected at the Infectious Diseases Unit, Karolinska University Hospital, Huddinge. From EDTA tubes, plasma was collected and stored in -80ºC, and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Plaque (Cytiva) and stored in liquid nitrogen. Samples from Cameroon were collected at the Yaounde University Teaching Hospital in Cameroon, as described in **paper V**. Samples from India were collected from a tertiary care ART Centre at the Government Hospital for Thoracic Medicine in Chennai, India, as described previously [55].

3.3 LATENCY CELL MODELS

As of now, there are still no recognizable markers for the identification of HIV-1 latently infected cells in humans. Therefore, the complexity of *ex vivo* driven analysis is hampered by the lack of material representing the diverse repertoire of persisting HIV-1. As a proxy, latency cell models have been created to represent a fraction of the reservoir within the body. These cell lines are generated from one clone, thereby comprising a homogeneous analytical tool. The advantage of these cell lines is their stability over time. However, overcoming the homogeneity will only be possible by verifying data in patient material or several alternative clones to show reproducibility in heterogeneous samples. Herein, our primary *in vitro* experiments have been performed using lymphocytic and monocytic latency cell models together with parental cell lines (**Table 1)** (**papers III and V**).

Table 1: Characteristics of cell lines used in **papers III and V**

Cell line	Linage	Properties	Ref
Jurkat	Lymphocytic	T cell leukaemia cell line	[230]
J-Lat 10.6	Lymphocytic	T cell leukaemia cell line Insert of transcriptionally latent HIV-1 *Nef* coding sequence is replaced by GFP Frameshift mutation in *env*	[231]
U937	Pro-monocytic	Diffuse histolytic lymphoma cell line	[232]
U1	Pro-monocytic	Diffuse histolytic lymphoma cell line Chronically infected with HIV-1 Minimal constitutive viral expression in absence of activation	[233]

3.4 OMICS ANALYSIS

3.4.1 Transcriptome analysis

Evaluation of the transcriptome identifies transcriptionally active genes during a specific timepoint, comprising all RNA in the cell. In **papers I and III**, we applied this method to measure the gene transcription activity in our HIV-1 cohort. The RNA sequencing was performed using Illumina HiSeq2500 at the National Genomics Infrastructure, Science for Life Laboratory in Stockholm, Sweden. This method is highly versatile for identifying differences between groups (sample clustering by principal component analysis (PCA), hierarchical clustering, and differential gene expression (DGE)), specific features of a group [ART specific genes (**paper III**)] and classifying detectable transcripts into their cognate pathway (Gene set enrichment analysis (GSEA)). Furthermore, digital cell quantification (DCQ) can be used to estimate frequencies of cell subpopulations using the deconvolution algorithm Estimating the Proportions of Immune and Cancer cells (EPIC) (**papers I and III**) in whole PBMCs.

3.4.2 Proteome analysis

While transcriptomic evaluations can give insight into what processes are active in cells the proteome holds yet another key to understand to what extent these transcripts are translated into proteins. In **papers I**, **II**, **and V** we used LC-MS/MS to identify the intracellular protein levels in our cohorts. The untargeted LC-MS/MS-based proteomics was performed at Proteomics Biomedium, Karolinska Institutet, using isobaric labelling technologies. Proteome analysis gives a higher level of physiological relevance as not all mRNAs are translated into proteins. Protein activity can also be dependent on secondary modifications and degradation times which cannot be identified using transcriptomic analysis. This method allows for identification of differences between groups (PCA, DGE, specific features in a group [EC specific genes (**paper I**)]), and classification of proteins into their cognate pathways. Furthermore, we used data from targeted proteomic profiling by OLINK® proximity extension assay for an immune-oncology/inflammation panel [226] (**papers II and IV**) and employed enzyme-linked immunosorbent assay (ELISA) (**paper II**) to validate the findings.

3.4.3 Metabolome analysis

Studies of metabolites allows for a deeper understanding of secreted proteins in plasma and supernatants. Metabolites can be considered as a chemical fingerprint that can elucidate underlying cellular processes indicative of the physiological state of cells. In **papers I**, **III**, **IV**, **and V**, we employed untargeted metabolomics to understand the global relative quantification of metabolic modulation as a discovery method. The untargeted metabolomics was performed using the HD4 Platform at Metabolon Inc in North Carolina, USA. Furthermore, in **papers IV and V** for validation, we employed targeted metabolomics aimed at AA, CMM, and TCA, respectively. Targeted metabolomics was performed using LC-MS/MS (AA) or GC-MS (CMM and sugars) at the Swedish Metabolomics Centre in Umeå, Sweden. This method was employed to get an absolute quantification of the metabolic pathways already known to be of interest in our cohort.

3.5 BIOINFORMATICS ANALYSIS

All bioinformatics analysis used in this thesis was performed in collaboration with the bioinformaticians in the group.

3.5.1 Integrative high-throughput data analysis

Omics data can be integrated to stratify acquired high throughput data on a more complex level. To identify features associated with the $PLWH_{EC}$ phenotype we integrated proteomic and transcriptomic datasets in **paper I**.

3.5.2 Genome-scale metabolic model and flux balance analysis

Genome scale metabolic model (GSMM) is a mathematical representation of all metabolic processes in a biological network. In **paper III**, we wanted to understand the host-pathogen interactions in the context of metabolic reprogramming. Therefore, we employed a context (disease-state) specific GSMM of biological networks in PBMCs. This model is based on a human-reference genome-scale metabolite model from Metabolic Atlas [234] in which transcriptomic data is integrated to make it context specific. Furthermore, flux balance analysis (FBA) was performed to determine the flux (rate of molecule turnover) value of each metabolic reaction in response to the disease [235].

3.5.3 Reporter metabolite analysis

Changes in metabolites are most often exerted through transcriptional regulation. Therefore, as a proxy of metabolic reprogramming we used reporter metabolite analysis on the transcriptomic data (**paper III**). This is an algorithm that can identify metabolites around where the major transcriptional changes occur [236]. For this analysis we used the human genome scale metabolic model as a reference and identified metabolites based on the transcription profile of the samples using the tool platform for Integrated Analysis of Omics data (Piano) [237].

3.5.4 Feature selection

In prediction models, feature selections are performed to reduce the number of input variables and improve the accuracy of the analysis. In **paper V**, we performed feature selection by random forest or partial least squares-discriminant analysis (PLS-DA). Random Forest is a machine learning classification algorithm where the result of multiple decision trees are

combined and determine the features to be selected for [238]. PLS-DA is a multivariate dimensionality reduction tool using a linear regression model to find relations between features to create a prediction model [239]. In **paper V**, the overlap of the results from both these methods were used to improve the accuracy of the feature selection in the cohort.

3.6 ANALYTICAL METHODS

3.6.1 Flow cytometry analysis

Flow cytometry was used for detection of metabolite receptor expression (**papers I and IV**), immune cell phenotyping (**papers II and IV**), ROS production (**papers III and V**), senescence markers (**paper III**), apoptosis (**paper III**), viability (**paper III**), purity of isolated cell populations (**papers II and IV**), HIF-1α levels (**paper I**), and latency reactivation (**papers III and V**) (**Figure 8**). All flow cytometry data was analysed using FlowJo (TreeStar Inc). The advantage of using flow cytometry analysis is the capacity to analyse a large scale of data at a single cell level. The results can further be explored using complex data analysis and representation, as described below.

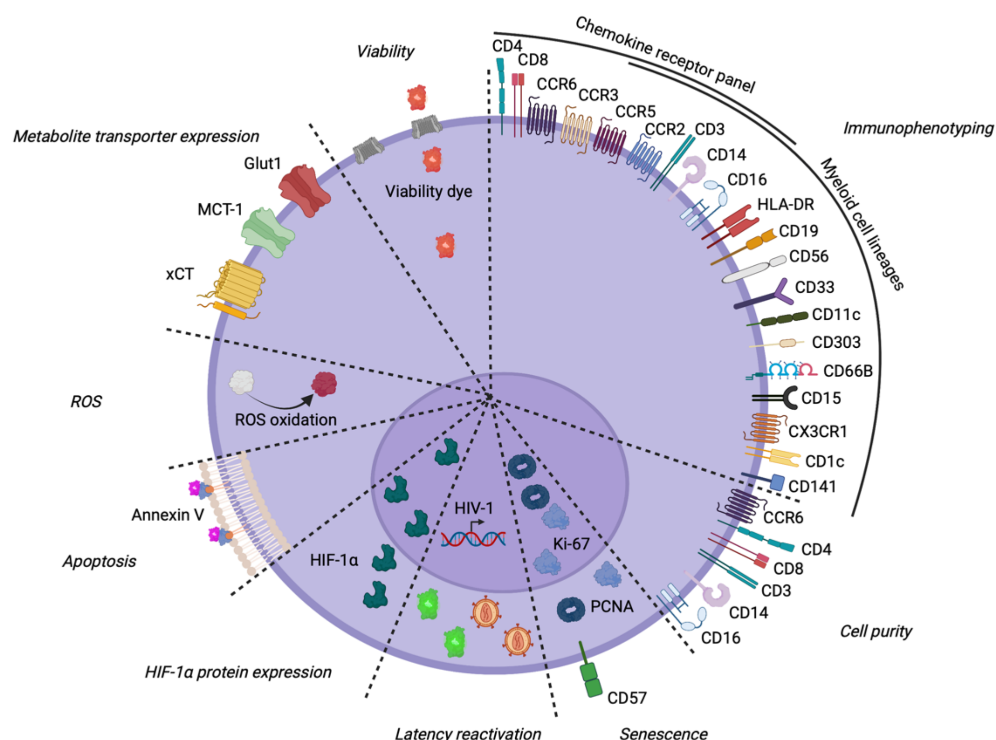

Figure 8: Methodological approaches for flow cytometry analysis used in the different manuscripts. Created using Biorender.com.

3.6.2 Immune cell isolation

Purification of specific immune cell subsets from total PBMCs allows for deeper downstream immune profiling than bulk analysis alone. In **paper IV**, conventional EasySep™ cell separation was used to isolate monocytes and CD4^{+} T cells. This protocol allows for robust isolation with high purity of traditional cell populations. The development of fluorescence-activated cell sorting (FACS) has furthered the field as it allows for more in-depth and specific cell type isolation not limited by conventional purification protocols. Therefore, in

paper II, we applied FACS to isolate and characterize the proteomic profile-specific cell populations of interest on the SONY cell sorter MA900 (SONY Biotechnology).

3.6.3 Western blot

To investigate total protein levels, we utilized western blot (**papers I**, **III**, **and V**). This robust method allows for relative protein detection in cell cultures and primary cells.

3.6.4 Immunofluorescence staining

Protein analysis still relies on immunofluorescence staining (IF) for accurate detection and localization of intracellular proteins. In **paper I**, this method was applied as the localization of HIF-1α is the determinant factor for activation. HIF-1α is a protein that upon activation translocates from the cytoplasm into the nucleus of a cell.

3.6.5 Polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) was used to identify transcriptional activation of some HIF-1α activated targets (**paper I**). Furthermore, in **papers II and III** we applied internally controlled qPCR (IC-qPCR) [97] to quantify the proportion of cells containing integrated HIV-1. IC-qPCR detects total HIV-1 5' long terminal repeats (LTRs) within the cells and can therefore be utilized as an approximation of cells carrying HIV-1. In **paper II**, we also used digital droplet PCR (ddPCR) to quantify more segments of HIV-1 DNA in the cells and intact proviral DNA assay (IPDA) [240] to determine what proportion of the integrated HIV-1 was carrying genetic deficiencies. The IPDA was performed by the Peter Svensson group at BioNut, Karolinska Institutet. In summary, these detection methods combined can give a good estimation of the quantity and state of the latent HIV-1 reservoir.

3.7 EXPERIMENTAL ASSAYS

3.7.1 Intracellular metabolite detection

To investigate the metabolic environment in PBMCs we measured intracellular glucose, lactate, and the glutamate/glutamine ratio in **paper I**. Similarly, in **paper V**, we measured intracellular metabolites in our latency model to see the effect ART had on the cellular metabolic environment. High throughput data were acquired on bulk PBMCs due to limitations of sample availability. Therefore, we utilized targeted metabolite detection methods for glucose, lactate, and glutamate to evaluate intracellular levels in $CD4^+$ T cells and monocytes by Promega Glo assays (**paper IV**).

3.7.2 Drug treatments

Initial cytotoxicity curves were created for drugs to identify concentrations not toxic to cells (**papers III and V**) [103]. We furthermore inhibited complex I-V of OXPHOS (**paper III**), glycolysis, and glutaminolysis (**paper V**) to see the effect on modulation of metabolic pathways in latency models. In **paper V** we also utilized ART regimens (tenofovir disoproxil fumarate (TDF) + lamivudine (3TC) + efavirenz (EFV) (TDF+3TC+EFV), and zidovudine (AZT) + 3TC + EFV (AZT+3TC+EFV)) prevalently used in low-income countries to model the effect of cell adaptation in PLWH on suppressive therapy.

3.8 REPRESENTATION OF COMPLEX DATA

Co-expression analysis of multiple markers (**paper II**) was determined by Boolean gating in FlowJo (Treestar Inc) and represented using Simplified Presentation of Incredible Complex Evaluations (Spice v6.0) (**Figure 9A**) [241]. For more complex data exploration, reduction, and visualisation two different dimension reduction techniques were used, namely t-distributed stochastic neighbour embedding (t-SNE) and uniform manifold approximation and projection (UMAP). t-SNE is based on an unsupervised and nonlinear algorithm of selected cytometric parameters to visualize the data in a reduced state (**paper II**) (**Figure 9B**) [242]. UMAP is a machine learning algorithm used to dimensionally reduce and visualize parameters in two-dimensional space while preserving the global structure of the data (**papers III**, **IV, and V**) (**Figure 9C**) [243]. Sankey plot was used in **paper V** to show the contribution of different metabolites to their cognate pathways (**Figure 9D**). In **papers I and V**, we also used Venn diagrams to show differing and overlapping characteristics between cohorts (**Figure 9E**) [244]. Network analysis and global association analysis network were used in **paper I-V** to represent the association or connection between detected variables from the high throughput data (**Figure 9F**). These networks were visualized using Cytoscape software.

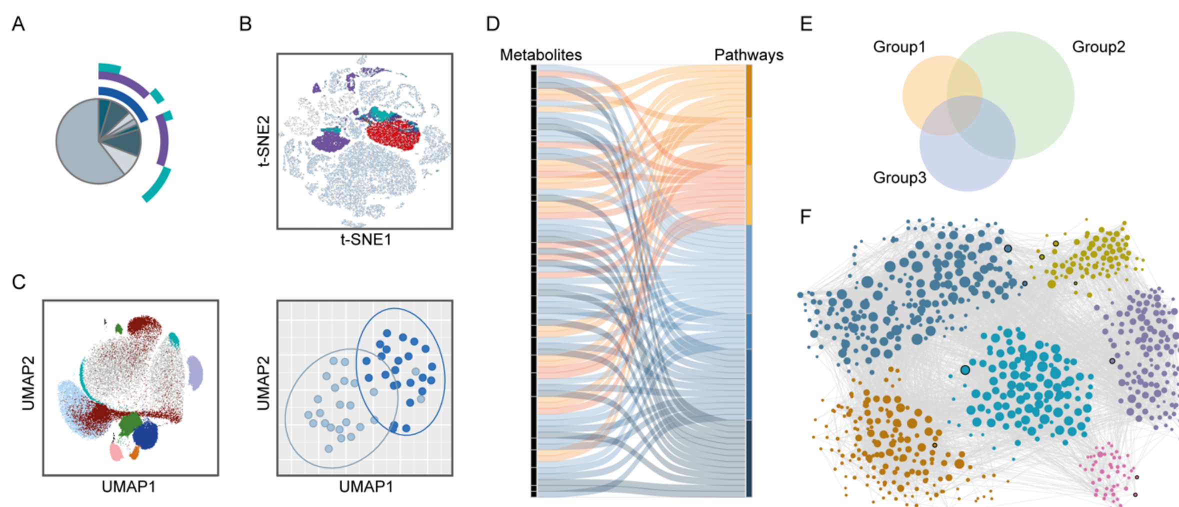

Figure 9: Representation of how complex data is presented in the thesis. (A) Spice plot adapted from **paper II**. (B) t-SNE plot adapted from **paper II**. (C) Two UMAP plots adapted from **papers IV and V**. (D) Sankey plot adapted from **paper V**. (E) Venn-diagram adapted from **paper I**. (F) Global association analysis network adapted from **paper IV**.

3.9 STATISTICAL ANALYSIS

All statistics was performed using Graphpad Prism or in R [245]. The choice of statistical methods was determined by the normality distribution of the dataset. For normally distributed data parametric tests were applied, such as students t-tests. For non-normally distributed data, non-parametric tests including Mann-Whitney U-test were used for unmatched samples and Wilcoxon-matched pairs signed rank test for matched samples. Correlation analysis was performed using the Spearman rank test due to lack of normality in the data. False discovery rate (FDR) was applied for correction of multiple comparisons to decrease risk of false positive. Effect size was calculated to compensate for relevant differences in standard deviations.

4 RESULTS AND DISCUSSION

Immune cell activity and function is orchestrated by a dynamic interplay between metabolic pathways and immunological signals, suggesting the importance of understanding both elements during HIV-1 pathogenesis and persistence. In an attempt to expand our knowledge within this multibranched area, this thesis was dedicated to studying the dysregulation of metabolism and immunity during HIV-1 persistence in a cohort with a controlled viral infection, either natural, $PLWH_{EC}$, or ART induced, $PLWH_{ART}$ (**Figure 10**). More specifically, in **paper I** we aimed to understand the characteristics of natural control of HIV-1 infection in $PLWH_{EC}$ by integrating proteomic and transcriptomic data. In **paper II** we investigated the immunophenotype in $PLWH_{EC}$ compared to $PLWH_{ART}$. In **paper III** we evaluated the metabolic modulation induced in $PLWH_{ART}$ and how it affects immune cell function compared to $PLWH_{EC}$. In the last two studies, **papers IV and V**, our main aim was to understand how long-term treatment affects the metabolic profile of cells in cohorts from Sweden, Cameroon, and India. Almost all studies in this thesis followed a data-driven exploration where omics data was used to lay the foundation for the laboratory investigations.

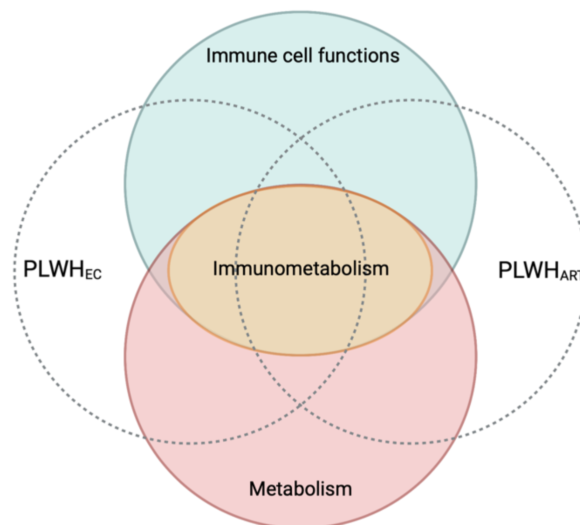

Figure 10: Overall aim of this thesis. Created using Biorender.com.

4.1 PHENOTYPE OF ELITE CONTROL

The characteristics of natural control of HIV-1 infection could potentially hold the key for an HIV-1 cure. Although, the discovery of multifunctional levels of control has introduced a complexity in understanding how this natural viral suppression is achieved. Earlier reports have shown a population-based heterogeneity in the $PLWH_{EC}$ group [226, 246] and in **paper I**, we identified a significant heterogeneity in $PLWH_{EC}$ between the sexes (**paper I, Figure 3A-C**). It is worth noting that the $PLWH_{EC}$ cohort is very small, although almost all known $PLWH_{EC}$ in Sweden were included. Therefore, as in most studies of $PLWH_{EC}$, one of the biggest limitations of all subsequent analyses was the sample size. We tried to utilize our resources to our best capacity, but in hindsight there are still many validatory experiments that could have strengthened the data. Even so, as mentioned in the introduction, extensive efforts have been put into understanding how natural control occurs, yet no consistent

mechanism of control has been found. Currently, data indicates that there are several parameters which differ at the individual level, promoting a natural control of HIV-1 infection. Alas, further studies are needed to unravel this complicated subject.

4.1.1 The immunophenotype of $PLWH_{EC}$ exhibits dysregulated T cell and monocyte compartments

Research on $PLWH_{EC}$ has shown reduced inflammatory levels indicative of normalized immunological functions [247]. Even so, we identified decreased levels of $CD4^+$ T cells and increased $CD8^+$ T cells compared to HC (**paper II, Figure 1A**). Disturbances in immune cell frequencies can be a consequence of cell death, function, or residency in distant organs and compartments of the body. Using digital cell quantification from transcriptomic data, no major differences were seen in other lymphocytic cell populations in $PLWH_{EC}$ compared to HC, while T-regs were significantly decreased compared to $PLWH_{ART}$ (**paper III, Figure 1A**). On immune cells, chemokine receptors play an important role in activation, function, and migration in normal state but also during HIV-1 infection [248]. In **paper II**, we detected decreased frequency and expression of CCR6 and decreased expression of CCR2 on $CD4^+$ and $CD8^+$ T cells in $PLWH_{EC}$ compared to $PLWH_{ART}$ and HC (**Figure 11**; **paper II, Figure C&D**). CCR6 is a homing marker for GALT expressed on some memory T cell subsets such as Th17 cells [249, 250]. T cells expressing CCR6 are more susceptible to active HIV-1 infection [251]. In $PLWH_{EC}$, $CD4^+$ T cells and Th17 cells are maintained in the gut mucosa and these individuals exhibit low markers of microbial translocation, indicative of a preserved mucosal barrier [252]. Therefore, the occurrence of tissue residency could be the causative factor for reduced $CCR6^+$ cells in the blood as compared to patients on ART. Proteomics on isolated $CD4^+CCR6^+$ cells also showed an enrichment of proteins involved in p53 signalling and apoptosis while $CD4^+CCR6^-$ cells showed an enrichment of IFN-γ response compared to $PLWH_{ART}$ (**paper II, Figure 5B**). Possibly, the enrichment of these pathways could sensitize $CD4^+CCR6^+$ cells to cell death mechanisms in $PLWH_{EC}$. p53 activity can also inhibit reverse transcription of the virus and suppress the trans-activator tat [253]. Consequently, the increased susceptibility of $CD4^+$ T cells to HIV-1 infection together with increased sensitivity to cell death signalling could possibly contribute to natural control by restricting HIV-1 replication and inducing apoptosis upon latency reversal.

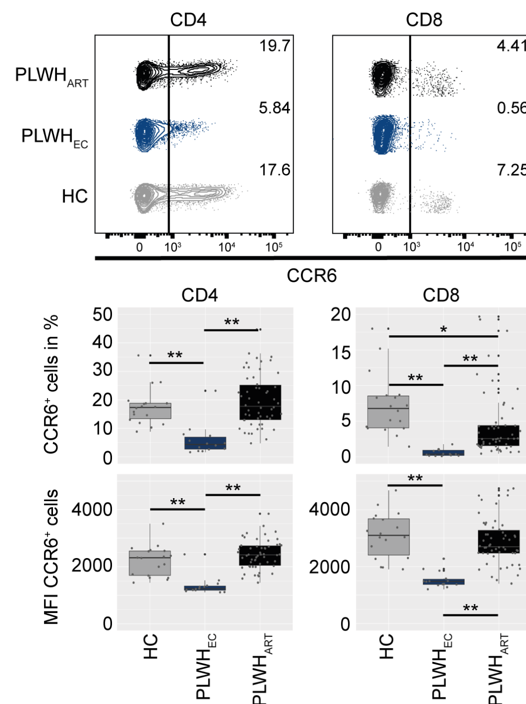

Figure 11: $CD4^+CCR6^+$ and $CD8^+CCR6^+$ cells are decreased in $PLWH_{EC}$ compared to HC. Adapted from **paper II**.

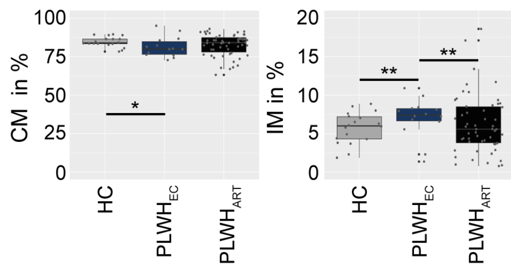

Figure 12: CM are decreased while IM are enriched in $PLWH_{EC}$ compared to HC. Adapted from **paper II**.

In **paper II**, we also evaluated the expression levels of monocytic cell populations: classical (CM), intermediate (IM), and non-classical (NCM) monocytes. In this cohort, we detected a decrease of CM, while IM were enriched in $PLWH_{EC}$ compared to HC (**Figure 12**; **paper II, Figure 2A&B**). The IM population has earlier been shown to be elevated in $PLWH_{EC}$ compared to HC [254]. The same study also demonstrated how the percentage of cells expressing CCR2 was decreased on CM in $PLWH_{EC}$, and they showed an overall increased response to LPS. Even so, the frequency of $CM^{+}CCR2^{+}$ cells did not differ, but we detected reduced expression levels of CCR2 on both CM and IM in $PLWH_{EC}$ compared to HC (**paper II, Figure 2D**). The CCR2 receptor is mainly involved in cell migration and eliciting an appropriate inflammatory response [255]. Furthermore, the IM subset is considered a more mature, pro-inflammatory subset with increased capacity for migration compared to CM [256]. Therefore, even as the level of inflammation is low [247], the phenotype of monocytes in $PLWH_{EC}$ suggests a higher activation of innate immune cells. Consequently, the inflammatory environment in $PLWH_{EC}$ might have a heightened surveillance for invading pathogens.

4.1.2 Altered metabolic environment in $PLWH_{EC}$ compared to HC

Alterations of the metabolic environment have been described during HIV-1 infection [257]. Knowledge of how this metabolic reprogramming occurs in $PLWH_{EC}$ is still limited. We started our exploratory analysis to identify features associated with the $PLWH_{EC}$ phenotype by integrating two levels of omics analysis in **paper I**. Both on a proteomic and proteo-transcriptomic integration level we saw an enrichment of glycolysis and HIF signalling in male $PLWH_{EC}$ compared to HC (**Figure 13A**; **paper I, Figure 1C&D; paper I, Figure 2D-F**). Our results indicated that the enrichment of HIF signalling was a result of increased translocation of HIF-1α and HIF-1β into the nuclei of lymphocytes, and a dysregulated transcription of HIF target genes in males (**Figure 13B**; **paper I, Figure 3A-H**). HIF-1α stabilization relies on low oxygen availability which is mostly seen in distant organs and body compartments [158]. In HIV-1 infection, the role of HIF signalling is a double-edged sword. On one hand, HIV-1 reactivation and LTR activity can be inhibited by HIF-1α stabilization thereby restricting viral production, as seen in $PLWH_{EC}$ [258]. This mechanism is also supported by the reduced tat-mediated HIV-1 transcription at lower oxygen levels [259]. On the other hand, HIV-induced HIF signalling sustains a feedback loop leading to viral replication and the release of extracellular vesicles, mediating an inflammatory response through activation of lymphocytes and macrophages [260]. Stabilization of HIF-1α by HIV-1 infection occurs through a HIF-1α association with the HIV-1 LTR. This is controlled by accumulation of ROS leading to deregulated transcription of host proteins [261]. Interestingly, $PLWH_{EC}$ did not exhibit any difference in ROS production indicative of a maintained redox homeostasis (**paper III, Figure 4B**). Overall, HIF signalling is an important regulator of immune cell activation; it constitutes an appropriate defence against viral pathogens while also being utilized by the virus for its own propagation. In the case of $PLWH_{EC}$, this unique feature could be a mechanism suppressing viral replication and spread.

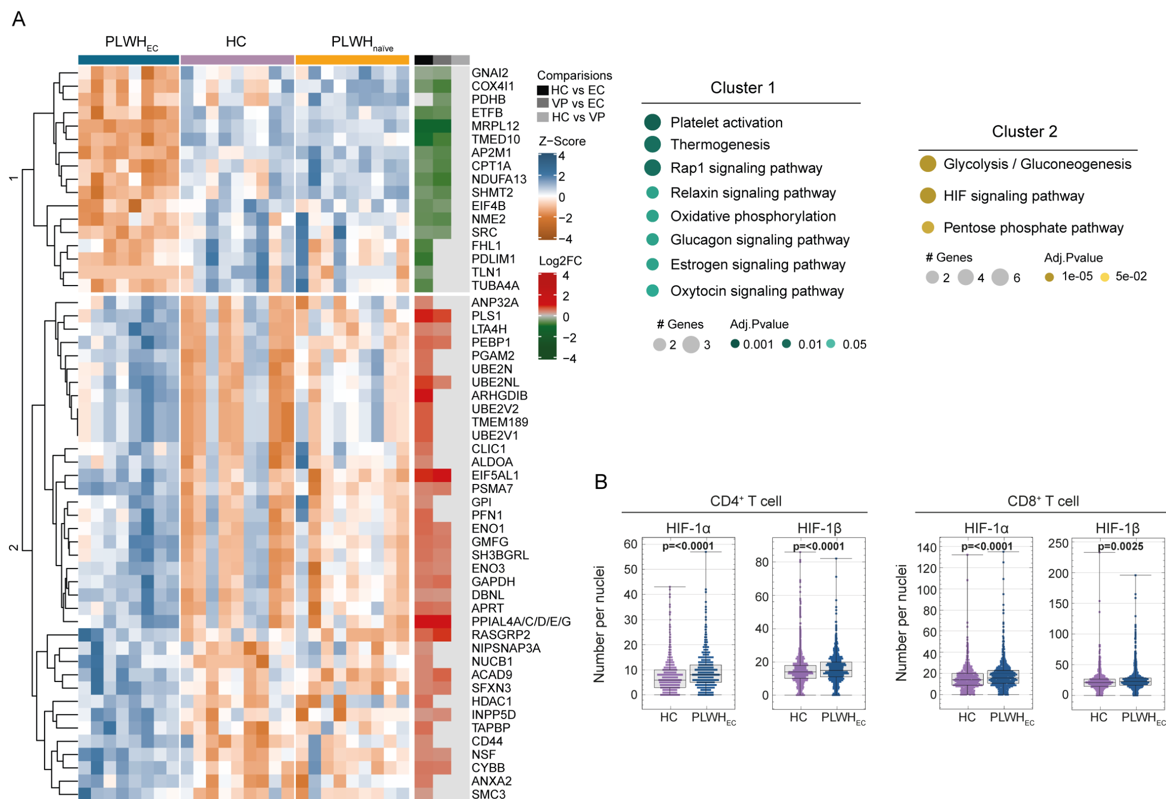

Figure 13: (A) Heatmap visualization of PLWHEC specific proteins with pathways decreased (cluster 1) and enriched (cluster 2) in PLWHEC compared to HC from proteo-transcriptomic integration. (B) HIF-1α and HIF-1β is enriched in the nuclei of lymphocytes in PLWHEC. Adapted from **paper I**.

Furthermore, the dysregulation of HIF target genes can mediate a broad range of functional outcomes, including metabolic reprograming, that possibly contribute to natural control of infection.

In line with dysregulated HIF signalling, glycolysis was enriched in male PLWHEC (**paper I, Figure 2D-F**). Many of the proteins involved in glycolysis can serve moonlighting functions by modulating inflammatory responses and aid in alternative cellular processes [262]. Additionally, antiviral properties have been attributed to some glycolytic enzymes e.g., GAPDH [263] and ENO1 [197, 198]. As metabolic reprogramming is controlled by HIF-1α and HIF-1β, and both glycolysis and HIF signalling were enriched in the PLWHEC group, it brought on the question of to what extent this mediated a metabolic dysregulation in the cohort. To study this, we measured the expression of a panel of key metabolite transporters and intracellular metabolite levels for glucose (Glut1), lactate (monocarboxylate transporter 1, MCT-1), and glutamate (cysteine/glutamate transporter, xCT)). We detected a decreased expression of Glut1 and MCT-1 in $CD8^+$ T cells and Glut1 in $CD4^+$ T cells in PLWHEC (**paper I, Figure 4A-C**). Reduced Glut1 expression can restrict viral replication [189], contributing to keeping HIV-1 in a resting state. After performing

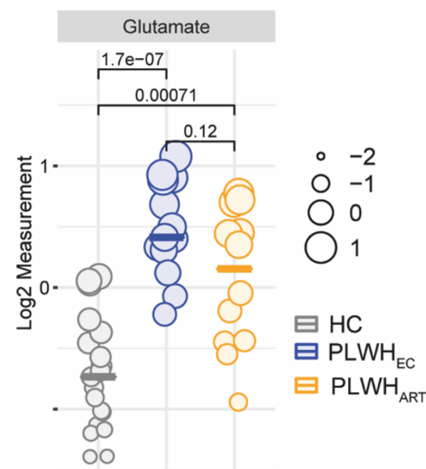

Figure 14: Plasma levels of glutamate are enriched in both PLWHEC and PLWHART compared to HC.

proteomics on $CD4^+$ T cells we saw an enrichment of proteins involved in OXPHOS and decreased glycolysis in $PLWH_{EC}$ compared to $PLWH_{ART}$ (**paper II, Figure 5B**). This is a metabolic profile generally associated with resting or naïve T cells [163]. Furthermore, we detected increased plasma levels of glutamate in $PLWH_{EC}$ compared to HC (**Figure 14)** indicating that glutaminolysis is an alternative fuel source used during controlled infection.

A loss of $PLWH_{EC}$ phenotype involves a metabolic shift to aerobic glycolysis prior to loss of control [204]. These patients simultaneously display deregulated mitochondrial activity, immune activation, and oxidative stress. Unsurprisingly, these events correlate to some extent with a virus induced metabolic shift for virion production [229], and support our data on decreased glycolysis in $CD4^+$ T cells from $PLWH_{EC}$. Still, the question remains what could induce this drastic change after long-term control of HIV-1. In our studies, even as we saw an enrichment of glycolysis in bulk PBMCs, glycolysis was decreased in $CD4^+$ T cells. The causative factor here could partly be what the $PLWH_{EC}$ group is compared with. On a scale with HC as a reference, the enrichment of glycolysis in $PLWH_{EC}$ is still relatively low compared to $PLWH_{ART}$ (discussed in next section). We also saw increased levels of intracellular glucose in $PLWH_{EC}$ compared to HC (**paper I, Figure 4D**). It is possible that the elevated glucose levels are a consequence of alternative cell types as the analysis was performed on bulk cells.

In conclusion, the $PLWH_{EC}$ group has many unique characteristics that could contribute to natural control of infection. It is unlikely that there is one feature facilitating this phenotype. Therefore, more research is needed to obtain a comprehensive understanding of how $PLWH_{EC}$ can be used as a model for a functional cure of HIV-1. Multi-omics layered or integrative approaches, such as those employed in **paper I**, have an advantage by increasing the complexity of the analysis. In the future, these types of methods can be a useful tool to stratify specific features of the $PLWH_{EC}$ phenotype on a higher level.

4.2 THE EFFECT OF HIV-1 INFECTION DURING SUPPRESSIVE THERAPY

HIV-1 infection contributes to major immunological alterations not fully normalized during suppressive ART. The most drastic changes occur during early ART to counteract the effect induced by viral replication [264]. At later stages of infection, these changes can create a persisting chronic inflammatory environment where the immunological aberrations have been well described [265]. With an ageing PLWH population, long-term suppressive therapy can possibly be associated with earlier onset of age-related diseases and co-morbidities [266]. However, viral suppression and immune reconstitution during long-term ART are not completely understood. Therefore, there is a need to understand the long-term effects in PLWH on suppressive ART and how it influences chronic inflammation.

4.2.1 Metabolic reprogramming in PLWH$_{ART}$ towards dysregulated AA and energy metabolism

During acute viral infections, the host metabolic environment is modulated to sustain the high energy demand needed for viral production. This involves increased aerobic glycolysis, similar to the metabolic reprogramming during T cell activation [166]. ART suppresses viral replication to undetectable levels in blood plasma and restores the virus-induced host-immunometabolic functions to some extent. Yet, some alterations persist even during long-term suppressive ART. In **paper III, IV, and V**, we explored to what extent the metabolic modulation occurs in PLWH$_{ART}$. In **paper III**, we identified an enrichment of OXPHOS and ROS pathway as the main difference in PLWH$_{ART}$ compared to PLWH$_{EC}$ on a transcriptomic level (**paper III, Figure 2C&D**). More specifically, OXPHOS complexes I, III, and IV were mainly affected (**Figure 15A**; **paper III, Figure 2E**). When HIV-1 infects CD4$^+$ T cells, both OXPHOS and glycolysis are upregulated [229]. Additionally, a consequence of mitochondrial respiration is the production of ROS that can disrupt the redox homeostasis and augment oxidative damage [267]. Thus, these two pathways are tightly interlinked. In our cohort, even as the ROS pathway was enriched, flow cytometry analysis did not detect any difference in ROS production in PLWH$_{ART}$ compared to PLWH$_{EC}$ or HC in lymphocytic cell populations (**paper III, Figure 4B**). However, increased ROS production was observed in patients with longer duration of ART (lnART) (>10yrs) as compared to those with shorter duration (sART) (<10yrs) in CD4$^+$ and CD8$^+$ T cells indicative of modulation of redox homeostasis (**Figure 15B**; **paper III, Figure 4D**). Some of the HIV-1 proteins can enhance

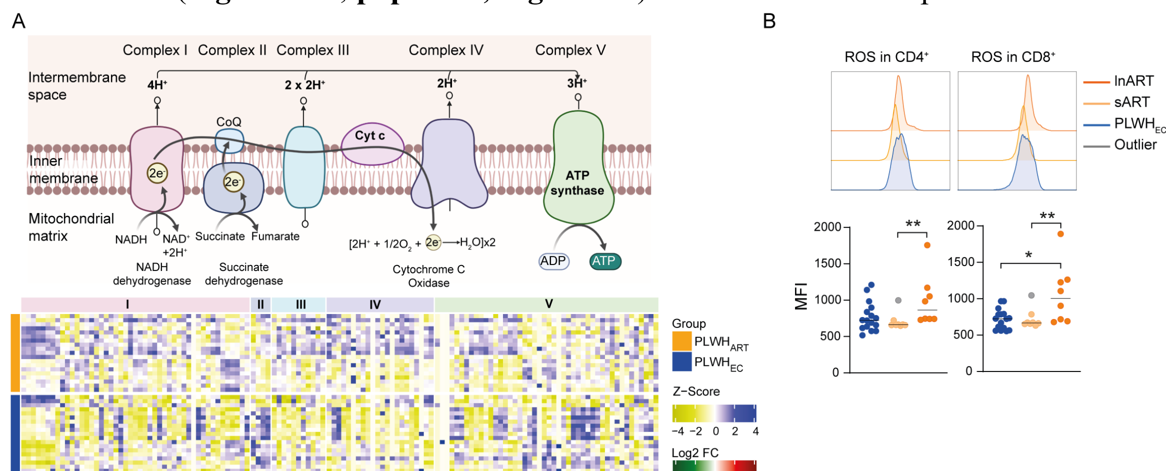

Figure 15: (A) OXPHOS is enriched in PLWH$_{ART}$ compared to PLWH$_{EC}$. (B) ROS levels are higher in lymphocytes from long-term ART treated (lnART) compared to short-term ART (sART) PLWH. Adapted from **paper III**.

ROS production leading to an increase in inflammatory processes [268]. Specifically in our cohort, most of the lnART patients also had earlier experience with the NRTIs zidovudine (AZT), stavudine (d4T), and/or didanosine (ddI). Toxicities associated with these early regimens can possibly contribute to long-lasting mitochondrial damage and oxidative stress [269, 270]. A recent study also identified dysregulation of mitochondrial functions in $PLWH_{ART}$, where poor energy generation possibly contributes to inflammageing during therapy [271]. Therefore, the modulated transcriptomic profile hints towards a transitory reprogramming of energy metabolism during suppressive ART.

To increase our understanding of the metabolic environment in $PLWH_{ART}$, we employed GSMM and flux balance analysis based on the transcriptomic data (**paper III, Figure 3B**). This analysis showed how most changes occurred in reactions belonging to AA metabolism, nucleotide, carbohydrate, and energy metabolism pathways like pyruvate, αKG, glutamate, and fructose-6-phosphate (**paper III, Figure 3C**). Collectively, this supports the data on dysregulated energy metabolism during ART.

Up to this point, most of our analysis in $PLWH_{ART}$ had focused on intracellular omics analysis to understand the metabolic environment during suppressive therapy. Therefore, to broaden our knowledge we employed plasma metabolomics in the Swedish InfCareHIV cohort in **paper IV**. Herein, both untargeted and targeted metabolic profiling showed increased levels of glutamate, pyruvate, and lactate in the plasma of $PLWH_{ART}$ compared to HC (**paper IV, Figure 1B&C; paper IV, Figure 2A**). Similarly, in **paper V**, plasma metabolites altered in $PLWH_{ART}$, in the Cameroon cohort, belonged to pathways including lipid biosynthesis, ROS signalling, and immune cell activation where AA, and specifically glutamate, played a central role (**paper V, Figure 1C**). Validating the data, targeted metabolomics from both the Cameroon and Indian cohorts were performed for AA metabolism. One limitation in need of mentioning is the cross-sectional design of the study limiting the results to association only. Even so, six altered AA were identified between cohorts in $PLWH_{ART}$ compared to HC (**paper V, Figure 3A**). Of these, glutamate was significantly increased while the other five (methionine, phenylalanine, threonine, valine, and tryptophan) were decreased in $PLWH_{ART}$ (**paper V, Figure 3B-G and J**). It is worth mentioning that glutamate levels were also enriched in $PLWH_{naïve}$ compared to HC in the Indian cohort but not in the Cameroon cohort (**Figure 16**; **paper V, Figure 3G**). One distinguishing characteristic between the $PLWH_{naïve}$ in the two cohorts was lower $CD4^{+}$ T cell counts in the Indian population. Severe depletion of $CD4^{+}$ T cells could potentially introduce detrimental effects on the metabolic environment and contribute to development of metabolic syndrome (MetS) [272].

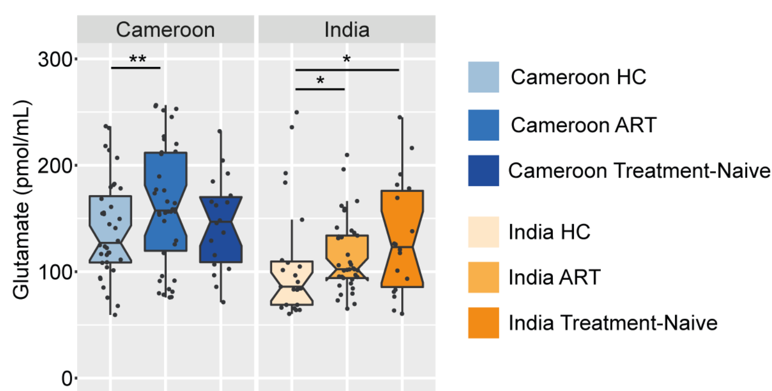

Figure 16: Targeted metabolomics showed enrichment of glutamate in $PLWH_{ART}$ compared to HC from both Cameroon and India. Additionally, glutamate is elevated in treatment naïve individuals from India. Adapted from **paper V**.

Glutamate is a versatile AA utilized in energy metabolism and AA biosynthesis [273]. One earlier study suggested that high plasma glutamate levels may contribute to loss of lymphocyte and macrophage functions [274]. Furthermore, excessive glutamate levels have been correlated to age-related neurodegenerative disorders due to excitotoxicity [275], and HIV-1 infection in macrophages can induce release of glutamate [209]. High extracellular glutamate levels have also been proposed as a contributor to neurological damage in HIV-1 infection [276]. In **paper V** we detected altered neurosteroid levels in $PLWH_{ART}$ in Cameroon and India, which is possibly involved in neurological complications (**paper V, Figure 2B**). Even so, glutamate levels were not changed after ART initiation (median 8 years therapy) in the Swedish InfCareHIV cohort (**Figure 17; paper IV, Figure 1E**), indicative of stable glutamate levels during therapy. On the other hand, we detected decreased levels of lactate while pyruvate levels increased in the same longitudinal cohort (**Figure 17; paper IV, Figure 1E**). These metabolites can influence inflammation where glutamate can contribute to neurological complications while lactate and pyruvate can play a role in protection of cellular stress by induction of a small-scale ROS production [213, 277]. Overall, our metabolic analysis shows a dysregulated AA metabolism in $PLWH_{ART}$. This phenomenon, earlier described during short-term ART (up to 36 months) [278, 279] and long-term ART (median 13 years) [272], therefore persists and possibly contributes to metabolic dysregulation and oxidative stress. Additionally, our studies on three separate cohorts hints towards an altered glutaminolysis. Earlier studies have also shown alterations in AA in $PLWH_{ART}$ with a central role of glutamate metabolism [280]. Late immune recovery during ART has also been attributed to dysregulation of glutaminolysis and lipoproteins [272, 281]. Central to glutamate metabolism is αKG that can modulate the epigenetic landscape to induce inflammatory signalling in cells [219]. Modulating this versatile system can possibly aid in restoration of immune cell functions during ART.

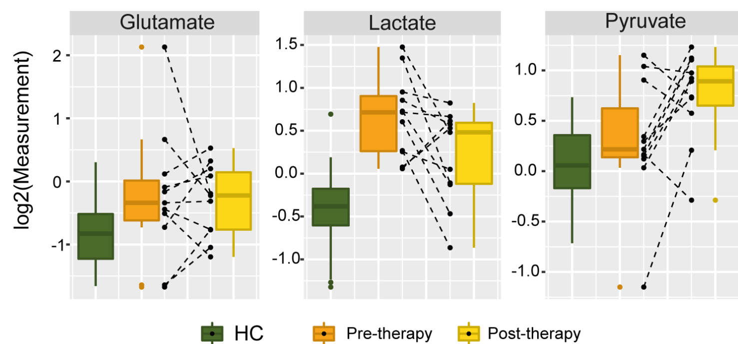

Figure 17: Untargeted metabolomics showing changes in glutamate, lactate, and pyruvate levels in PLWH pre- and post-therapy (median 8 years). Adapted from **paper IV**.

To understand the metabolic modulation on a cell-type specific level, we employed the same flow cytometry panel for metabolite transporters as in $PLWH_{EC}$. The analysis showed that changes in most receptor expression occurred on CM with an increase in $Glut1^{+}$ CM and MCT-1 expression on CM in $PLWH_{ART}$ (**paper IV, Figure 3C&D**). During monocyte and macrophage activation, Glut1 expression is increased to facilitate the shift towards elevated glucose metabolism [282]. Others have also described how activated monocytes expressing Glut1 are enriched during HIV-1 infection [283]. In the same analysis, we did not see any difference in $CD4^{+}$ or $CD8^{+}$ T cells (**paper IV, Figure 3C&D**). These data indicate that the major metabolic modulation in PBMCs is possibly a consequence of the monocytic cell

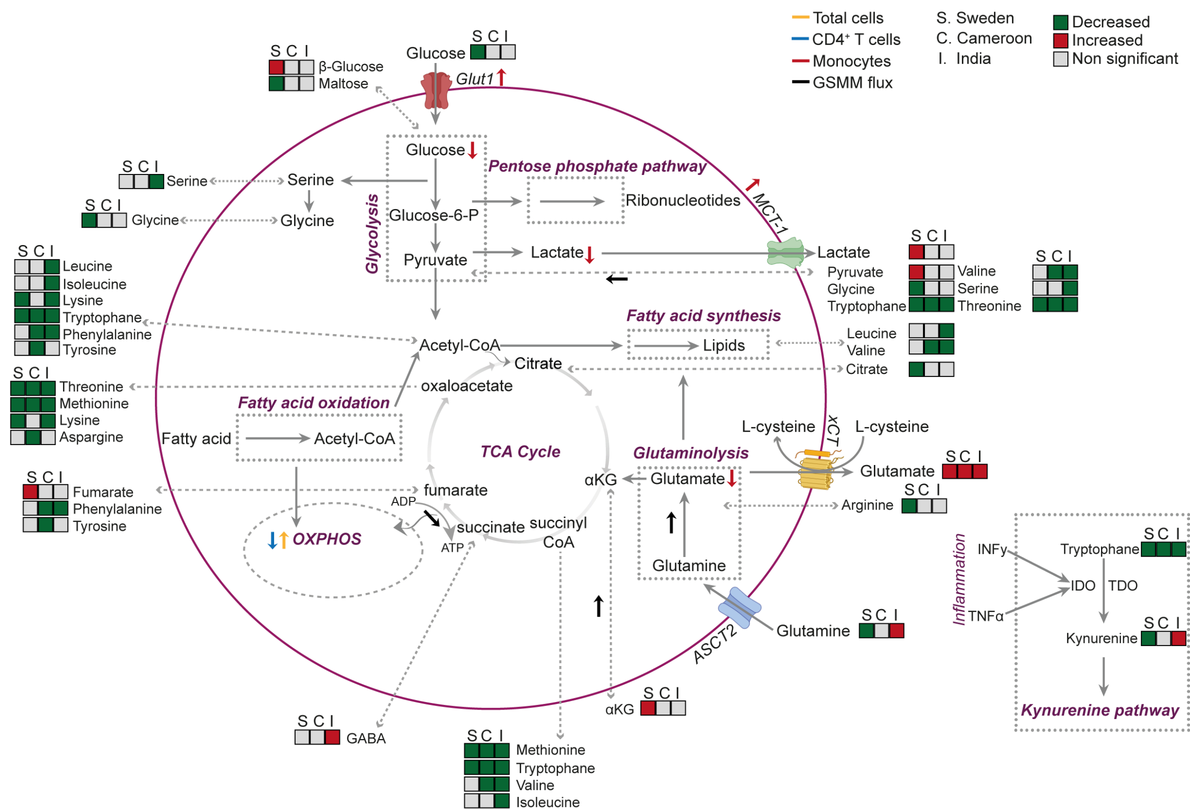

Figure 18: Proposed metabolic modulation of cells detected in PLWH$_{ART}$ compared to HC in cohorts from Sweden, India, and Cameroon in **paper II-V**. Yellow arrows described differences detected in total cells while results from monocytic cell populations are marked in red, and CD4$^+$ T cell populations in blue from the Swedish cohort. Additionally, relevant flow of information from genome scale metabolic model (GSMM) and flux balance analysis from the Swedish cohort is marked in black. The boxes represent the targeted plasma metabolomics from Sweden (S), Cameroon (C), and Indian (I) cohorts. The figure was adapted from **paper V**.

populations. Therefore, we isolated CD4$^+$ T cells and monocytes to measure intracellular metabolite levels in our cohort (**paper IV, Figure 3E**). In these cell subsets, we detected lower levels of glutamate, lactate, and glucose in monocytic cell fractions in PLWH$_{ART}$ compared to HC while no significant differences were seen in lymphocytes (**paper IV, Figure 3F&G**). Glucose mediated oxidative stress can drive the inflammatory response of monocytes/macrophages expressing high levels of Glut1 and ROS [282]. In our cohort, we detected increased ROS levels in CM from PLWH$_{ART}$ compared to HC (**paper III, Figure 4B**). Consequently, aberrant monocyte metabolism and function could drive inflammatory processes during suppressive therapy (**Figure 18**).

In the papers included in this thesis, we focused our targeted metabolomics analysis on the PLWH$_{ART}$ group. However, we have earlier published data on plasma metabolomics from PLWH$_{EC}$ [247]. Therefore, for this thesis we used batched correction on normalized data to compare the difference between the two groups. From this analysis, a total of 39 metabolites were significantly altered (adjusted $p<0.05$) between the PLWH$_{EC}$ and PLWH$_{ART}$. Of these, 23 metabolites were classified as lipids, 7 as AA, 4 as nucleotides, 2 as peptides, 2 co-factors and vitamins, and one as a carbohydrate. Among these metabolites, serine was decreased in PLWH$_{ART}$ while both cholate and pyruvate were increased compared to PLWH$_{EC}$ (**Figure**

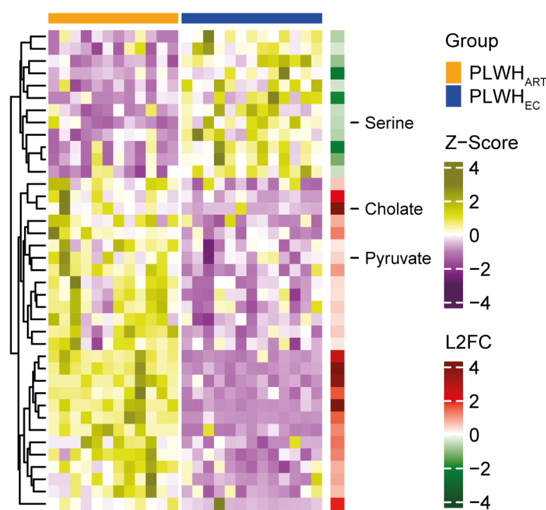

Figure 19: Differential expression of metabolites in $PLWH_{ART}$ compared to $PLWH_{EC}$ from untargeted metabolomics.

19). Although the relevance of these metabolites in $PLWH_{EC}$ is not yet elucidated it shows that there are unique metabolic markers that differentiate the metabolic environment during natural compared to ART induced control of HIV-1.

4.2.2 Immunophenotyping of $PLWH_{ART}$ shows dysregulated chemokine receptor expression on myeloid cell subsets

HIV-1 infection can be considered an immunomodulatory disease, partially as a consequence of the infection of $CD4^+$ T cells and their associated decline during viremia. Despite implementation of ART, these immunological aberrations do not normalize to the same level as HIV-1 negative individuals [284-286]. In our cohort, $PLWH_{ART}$ exhibited reduced $CD4^+$ T cells and increased $CD8^+$ T cell frequency compared to HC, as expected (**paper II, Figure 1A; paper III, Figure 3SB**). Recuperation of $CD4^+$ T cells to normal levels is faster with early initiation of therapy [64]. Initiation of ART with very low $CD4^+$ counts (<200cells/mm^3) can limit the recovery [287], although most patients do recuperate the initial $CD4^+$ T cell loss. In the Swedish InfCareHIV cohort this is a concern for ~2% of all patients that never reach $CD4^+$ T cell counts above 200cells/mm^3 despite viral suppression. Using digital cell quantification from transcriptomic data no major differences were seen in lymphocytic subpopulations in $PLWH_{ART}$ compared to HC (**paper III, Figure 1A**). When characterizing the receptor expression pattern in **paper II**, we saw that $PLWH_{ART}$ resembled the HC group on lymphocytic cell populations with an increased proportion of $CD4^+CCR6^+$, $CD8^+CCR6^+$, $CD4^+CCR2^+$, and $CD8^+CCR2^+$ T cells compared to $PLWH_{EC}$ (**paper II, Figure 1C&D**). Even so, the frequency of $CD8^+CCR6^+$ cells was still lower in $PLWH_{ART}$ compared to HC (**paper II, Figure 1D**). Proteomic analysis of $CD4^+CCR6^+$ cells showed reduced expression of proteins involved in apoptosis and p53 signalling in $PLWH_{ART}$ compared to $PLWH_{EC}$ (**paper II, Figure 5B**). Based on the receptor profile we investigated, this data shows how $PLWH_{ART}$ resemble HC on lymphocytic cell populations. However, as $PLWH_{ART}$ have a high impairment of the mucosal barrier and reduced $CD4^+$ T cell levels in GALT [288], normal frequencies seen in blood could be a consequence of reduced dissemination to distant tissues. The activity, function, and senescent profile on these cell populations would have been interesting to evaluate.

In **paper II**, we identified that longer suppressive therapy (>10 years) reduced expression of CCR2 on all three monocytic cell populations compared to shorter therapy (<10 years) (**paper II, Figure 3B-D**). Furthermore, the percentage of $CD8^+$ T cells expressing CCR2 or CCR6 increased while IM expressing CCR3 or CCR5 decreased with longer treatment duration (**paper II, Figure 3B-M**). As our initial analysis evaluated the immunophenotype of lymphocytes during suppressive therapy it raised the question of changes in alternative cell linages. Especially since the main metabolic reprogramming in our cohort occurred in the monocytic cell population, as mentioned above. Therefore, we aimed to characterize cells

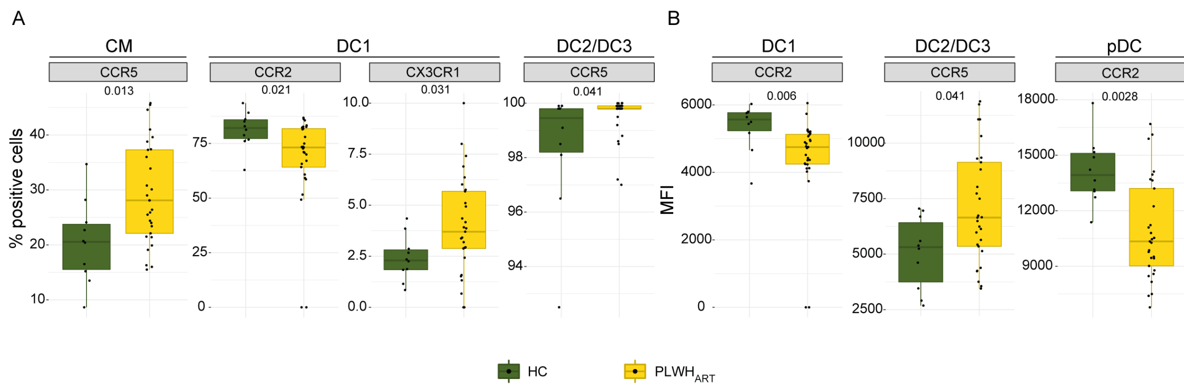

Figure 20: Chemokine receptor expression on myeloid cell lineages in PLWHART compared to HC. (A) Cells expressing CCR5, CCR2, and CX3CR1 within myeloid cell lineages in %. (B) Mean fluorescent intensity of CCR2 and CCR5 on dendritic cell (DC) cell populations. Adapted from **paper IV**.

from the myeloid cell lineage (**paper IV, Figure 4A**) and some key chemokine receptors, CCR2, CCR5, and CX3CR1, that mediate trafficking of immune cells. No difference in the frequency of cell populations was observed between PLWHART and HC (**paper IV, Figure S2B**). However, the main differences were an increase in CCR5^{+} CM and DC2/DC3 while CX3CR1^{+} DC1 was increased and CCR2^{+} DC1 was decreased (**Figure 20A**; **paper IV, Figure 4D**). Furthermore, CCR2 expression was decreased on DC1 and pDC while CCR5 expression was increased on DC2/DC3 (**Figure 20B**; **paper IV, Figure 4E**). As CCR5 is one of the main co-receptors used for HIV-1 entry into the cell, dependency for M-trophic virus has also been described [91], this poses the question of the relevance of CCR5 in the broader myeloid subpopulations. In combination, increased CCR5 expression on CM and DC2/DC3 could result in increased HIV-1 susceptibility. Receptor-ligand interaction of the CX3CR1 receptor is involved in cell homeostasis and tightly linked with survival of immune cells [289]. Therefore, the reduced expression of CCR2 and CX3CR1 could affect the responsiveness to DC migration and decrease the capacity to counteract cell death mechanisms. In the same cohort, we also detected elevated levels of pro-inflammatory cytokines (**paper IV, Figure 2C**). Two of these, macrophage inflammatory protein 3 alpha (MIP-3α) and monocyte chemotactic protein 3 (MCP-3), both play a role in monocyte inflammatory responses and recruitment of monocyte and macrophages to the site of inflammation [290, 291]. Therefore, the heightened inflammatory response in PLWHART could contribute to dysregulated myeloid cell functions during suppressive HIV-1 infection.

In **paper IV**, we evaluated inflammatory markers in the PLWHART. Additionally, in an earlier publication from the group we assessed the same inflammatory panel in PLWHEC [247]. To understand differences between these two cohorts we compared the results from the targeted proteomic analysis. In order to compare this data, we used batch correction on normalized data. From the 92 inflammatory markers used, eight were significantly increased (adj p<0.1) in PLWHART compared to PLWHEC

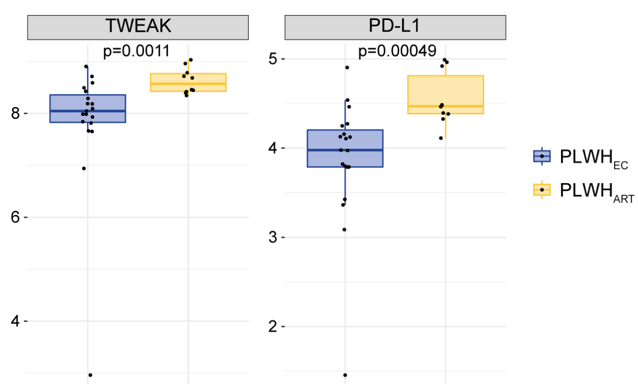

Figure 21: Batch correction of normalised data showing enrichment of inflammatory markers TWEAK and PD-L1 in PLWHART compared to PLWHEC.

including, Angiopoietin-1 receptor (TIE2), phosphatidylinositol-glycan biosynthesis class F protein (PIGF), natural cytotoxicity triggering receptor 1 (NCR1), pleiotrophin (PTN), vascular endothelial growth factor A (VEGF-A), and macrophage colony-stimulating factor 1 (CSF-1). Two of these enriched inflammatory proteins were programmed cell death 1 ligand 1 (PD-L1) and tumour necrosis factor (ligand) superfamily, member 12 (TWEAK), both involved in cell death mechanisms (**Figure 21**). An enrichment of these ligands indicates higher activation of cell death responses in $PLWH_{ART}$ compared to $PLWH_{EC}$. Additionally, as all markers were elevated in $PLWH_{ART}$ it indicates increased inflammatory levels in $PLWH_{ART}$ compared to $PLWH_{EC}$.

4.3 MODELLING AND DETECTION OF PERSISTENT HIV-1

As mentioned in the introduction, HIV-1 persists during controlled infection. The latently infected cells are not affected by ART and the complete mechanisms regulating the transcriptional activity of latent HIV-1 are still unknown. Identification of latently infected cells, obtained from humans, is still not possible due to the lack of markers. Therefore, latency models are a useful tool to study the unique characteristics of this subset of infected cells. However, the homogeneity of cell cultures is one of the main limitations as they can never completely represent the diverse pool of infected cells in the human body. To overcome these limitations, a lot of efforts have been put into creating primary latency cell models [292, 293]. These primary cell culture models can be informative, but no single cell culture model alone will be sufficient to completely understand the characteristics of persisting HIV-1 [294]. Consequently, we decided to study HIV-1 latency with established latency models in our experimental design and then quantify the viral reservoir *ex vivo*.

4.3.1 Inhibition of metabolic pathways in latency cell models

In **paper III** and **V,** we used lymphocytic (J-Lat 10.6) and pro-monocytic (U1) latency cell lines to model the effect of our data-driven results *in vitro*. These established cell lines can both be readily activated using the PKC agonist prostratin (**paper V, Figure 4C&D**). In **paper III**, we detected an enrichment in OXPHOS in $PLWH_{ART}$ and increased ROS in lymphocytes during long-term suppressive ART compared to short-term. Therefore, we asked ourselves what role the different complexes of OXPHOS play in latent cells from a lymphocytic cell lineage. Using pharmacological inhibition of all five OXPHOS complexes we saw that only inhibition of OXPHOS complex III increased apoptosis specifically in the latency model, while inhibition of complex IV increased HIV-1 reactivation (**Figure 22**;

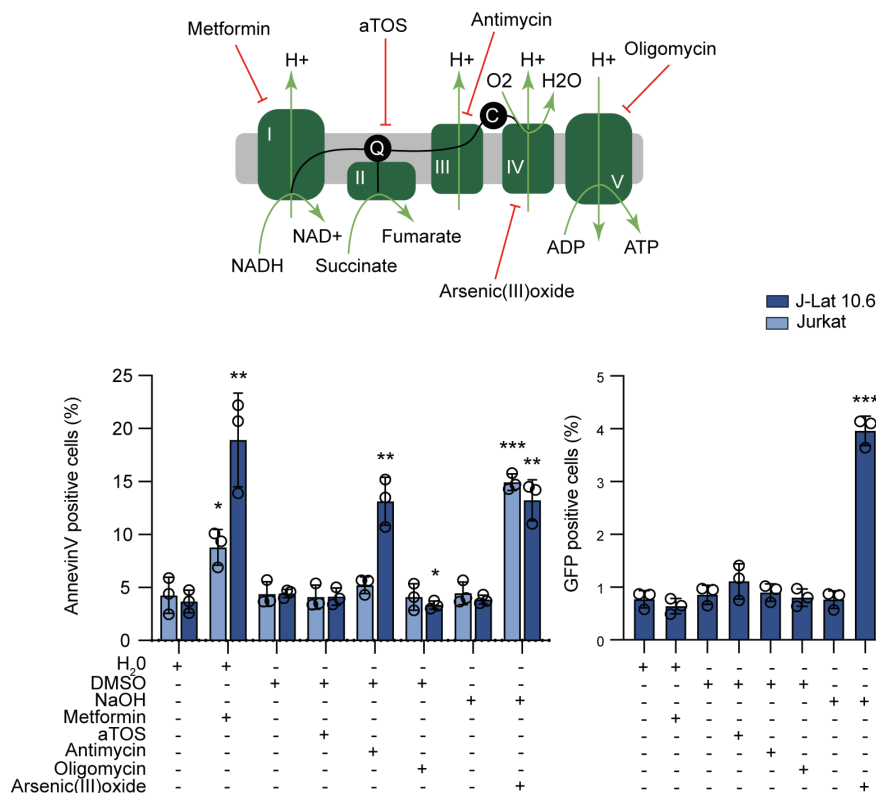

Figure 22: Inhibition of OXHPOS complexes in lymphocytic latency cell model J-Lat 10.6 together with parental cell line Jurkat. Figure shows induction of apoptosis by AnnexinV staining and reactivation from latency (GFP). Adapted from **paper III**.

paper III, Figure 5C&D). Even as inhibition of complex I increased apoptosis in both cell lines, the effect was larger in J-Lat 10.6 cells compared to the parental cell line (**Figure 22**; **paper III, Figure 5C**). Earlier studies have shown an induction of senescence in human fibroblasts during OXPHOS inhibition [295]. Therefore, we evaluated how this inhibition affected markers for senescence and replication. The senescence marker CD57 showed a general increase in our latency model compared to the parental cell line (**paper III, Figure S5A-C**). Furthermore, inhibition of OXPHOS complex IV decreased cell proliferation (Ki-67) while increasing DNA damage (H2A.X (S139)), indicative of a senescent profile (**paper III, Figure 5E&H**). Inhibition of glutaminolysis has earlier been shown to specifically remove senescent cells through senolysis [296]. Possibly, this inhibition could sensitize cells towards pharmacological induced cell death. Collectively, these data suggest a role of OXPHOS in regulation of latency and senescence in lymphocytic cell lineages.

In **paper IV** and **V**, we observed altered AA metabolism in three separate cohorts of $PLWH_{ART}$ indicative of a dysregulated energy metabolism during suppressive therapy. Therefore, in **paper V**, we used our latency cell models to study metabolic dysregulation during HIV-1 persistence. Initial proteomic analysis showed an enrichment of TCA cycle and biosynthesis of AA in our latency cell models compared to the uninfected parental cell lines (**paper V, Figure 4A**). We thereafter inhibited glycolysis (2-DG) and glutaminolysis (DON) to study the effect on the latent virus. The latency cell models were more sensitive towards inhibition of glycolysis and glutaminolysis compared to parental cell lines, while inhibition of glutaminolysis alone increased latency reversal in the U1 cells (**Figure 23**; **paper V, Figure 4B-D**). The protein profile of these U1 cells during inhibition of glutaminolysis showed decreased expression of proteins in OXPHOS compared to control (**paper V, Figure 4G&H**). This stipulates the role of glutaminolysis in latently infected monocytes. Furthermore, we evaluated how the metabolic modulation is affected during ART using two regimens prevalently used in low- and middle-income countries (TDF+3TC+EFV and AZT+3TC+EFV). A similar trend for intracellular glucose, glutamate, and lactate was seen independently of ART regimen or control. This indicates that the ART regimen does not affect the inhibition of glycolysis or glutaminolysis (**paper V, Figure 5C**). Latency reversal also decreased glucose and increased glutamate levels compared to controls. In conclusion, our results suggests a role for metabolic reprogramming during activation from latency.

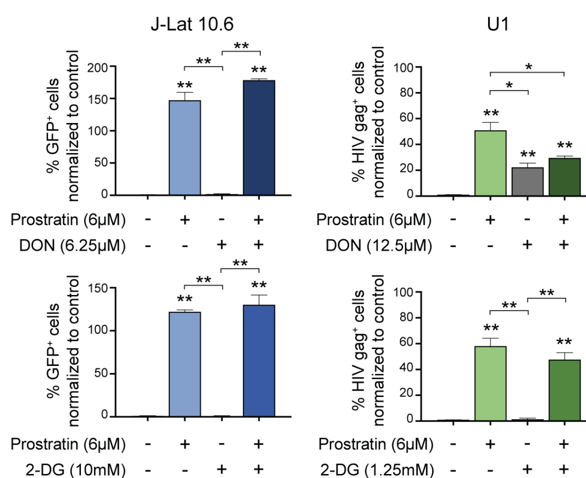

Figure 23: Inhibition of glycolysis (2-DG) or glutaminolysis (DON) with or without latency reversal agent prostratin in lymphocytic cell model J-Lat 10.6 and pro-monocytic cell model U1. Adapted from **paper V**.

4.3.2 The HIV-1 reservoir is lower in $PLWH_{EC}$ compared to $PLWH_{ART}$

The HIV-1 reservoir is the main obstacle to an HIV-1 cure. After detecting an immunometabolic reprogramming during controlled infection, we asked ourselves what the characteristics are of the HIV-1 reservoir in our cohorts. Firstly, we saw that length of treatment did not affect the proportion of latently infected cells when patients with >10 years of ART were compared to those with <10 years of ART (**paper II, Figure 4B**). This suggests that during suppressive ART, the relative size of the reservoir is not affected after the initial two phases of HIV-1 DNA decline earlier described [63, 297]. Comparing the two cohorts, $PLWH_{EC}$ exhibited a decreased proportion of integrated HIV-1 DNA compared to $PLWH_{ART}$, in line with earlier studies (**Figure 24A**; **paper III, Figure 2A**; **paper II, Figure 4A**) [298]. One of the mechanisms contributing to natural control has been proposed to be the integration site of the virus [44]. In this manuscript, Jiang *et al.* proposed that $PLWH_{EC}$ have a larger proportion of integrated virus in chromatin regions carrying repressive histone modifications. We did not detect any difference in intact provirus between the groups although the presence of 3'deletion/hypermutation was reduced in $PLWH_{EC}$ compared to $PLWH_{ART}$ (**Figure 24B**; **paper II, Figure 4D**). It is worth noting that the amount of intact provirus was low in both $PLWH_{EC}$ and $PLWH_{ART}$. Earlier studies have suggested that $PLWH_{EC}$ have a reduced proportion of intact provirus [44, 299]. Even so, these studies, and ours, did not demonstrate any decrease in the ratio of intact to total HIV-1 DNA suggesting that the reservoir in $PLWH_{EC}$ is not only made up of defective virus (**Figure 24C; paper II, Figure 4E**). The immune activating capacity of the 3' deletion clone could possibly explain its decline and the reduced reservoir if the negative selection is more potent in $PLWH_{EC}$ [300, 301]. Therefore, these data support earlier publications showing how the smaller reservoir in $PLWH_{EC}$ is not a consequence of a larger fraction of replication incompetent provirus compared to $PLWH_{ART}$. As other groups have proposed, low replication of the reservoir in $PLWH_{EC}$ is probably a consequence of natural control of infection rather than a causative factor [302]. Additionally, activation of HIF signalling can reduce HIV-1 LTR activity during normoxic conditions [258] strengthening the concept of HIF mediated control of HIV-1 persistence in $PLWH_{EC}$, as proposed in **paper I**.

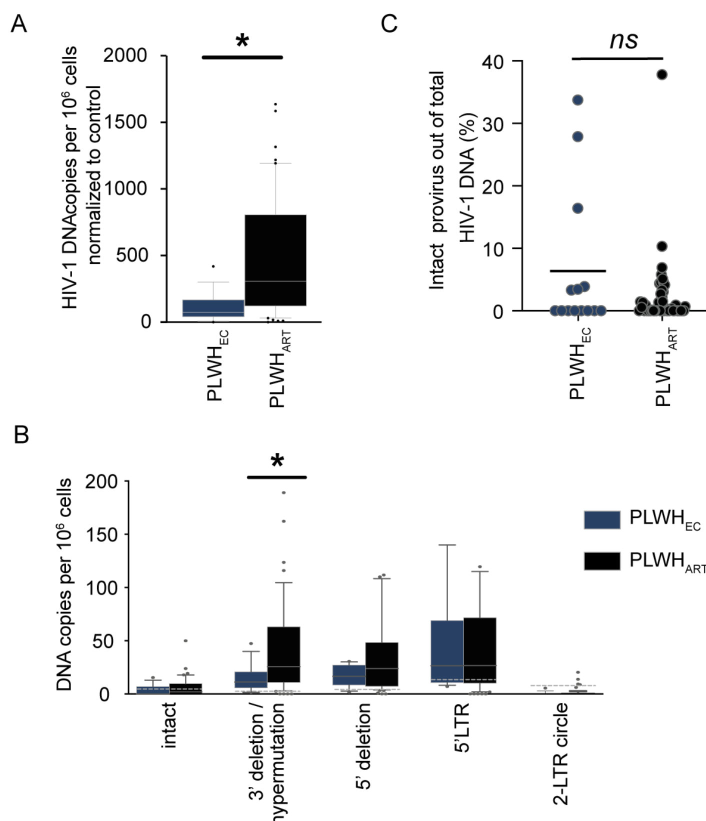

Figure 24: HIV-1 DNA quantification in $PLWH_{ART}$ compared to $PLWH_{EC}$ using IC-qPCR (A) and ddPCR (B). (C) Percentage of intact out of total detected HIV-1 DNA. Figure was adapted from **paper II**.

5 CONCLUDING REMARKS AND FUTURE DIRECTIONS

The understanding of how HIV-1 persistence is regulated during suppressive ART has increased during the last couple of years, yet new knowledge has only indicated a more profound complexity than originally considered. When this work started, the tight network of immunometabolic regulation was emerging as an important factor for cellular functional responses during HIV-1 infection. In this thesis, we aimed to explore the immunological aspects of controlled HIV-1 infection (either natural or induced by ART) and the role of metabolism during HIV-1 persistence.

Natural control of HIV-1 is a complex subject and of a heterogeneous nature where a combination of factors collectively contributes to the control. In **paper I**, we identified an enrichment of glycolysis and HIF signalling as unique features of the male $PLWH_{EC}$ phenotype. Although not a sole mechanism, this possibly contributes to repressing HIV-1 transcription through HIF activation [258]. Additionally, the unique metabolic profile of bulk PBMCs and dysregulated metabolite uptake and secretion could potentially be a universal mechanism restricting infection/HIV-1 reactivation, or merely a consequence contributing to low level of inflammation in $PLWH_{EC}$. The lack of similarity between the sexes is another factor contributing to the heterogeneity of the mechanisms of HIV-1 control. Differential immune cell functions between the sexes can be a consequence of insufficient X chromosome inactivation, hormonal levels, or nutritional intake [303]. These elements may facilitate regulatory functions of HIV-1 through alternative mechanisms between the sexes, but it might not always affect the phenotypic outcome, as seen in the similar metabolic profile in **paper I**. Future studies need to address this for the underlying experimental setup. Additionally, the unique receptor expression profile of lymphocytic cell populations in $PLWH_{EC}$ indicates that these cells are more potent at activating cell death mechanisms, as described in **paper II**. This may be a means to clear out infected cells upon latency reactivation. Depletion of these $CCR6^+$ cells in the circulation of $PLWH_{EC}$ could be a consequence of tissue residency in GALT to counteract the dysregulated mucosal barriers induced by HIV-1. CCR6 is a marker on some memory T cell subsets which include Th17, γδ T, and NKT cells, and is important at driving inflammatory responses in blood and tissues [250]. Limitations in sample availability restrained validation and further examination of these theories in our analysis. However, further studies examining the immunophenotype of the $CD4^+CCR6^+$ cells and their functional capacity to elicit an immune response and induce cell death mechanisms could help shed light on their relevance during HIV-1 infection.

Our comparative studies between $PLWH_{EC}$ and $PLWH_{ART}$, as discussed above, have shown large discrepancies between both immune cell function, inflammation, and metabolism (**Figure 25)**. From **paper III**, our main conclusion was that $PLWH_{ART}$ have a system level enrichment of OXPHOS, mainly complexes I, III, and IV, compared to $PLWH_{EC}$, which possibly plays a role in HIV-1 reservoir dynamics. Earlier studies have shown that OXPHOS is enriched during HIV-1 infection, correlating to disease progression and that mitochondrial functions and cellular homeostasis are disrupted in $PLWH_{ART}$ [229, 271]. Possibly, these mechanisms accumulate oxidative damage during suppressive ART which could be a leading consequence of earlier onset of age-related diseases in some patients. The $PLWH_{EC}$ group,

on the other hand seems to maintain redox homeostasis, thereby minimizing levels of inflammation.

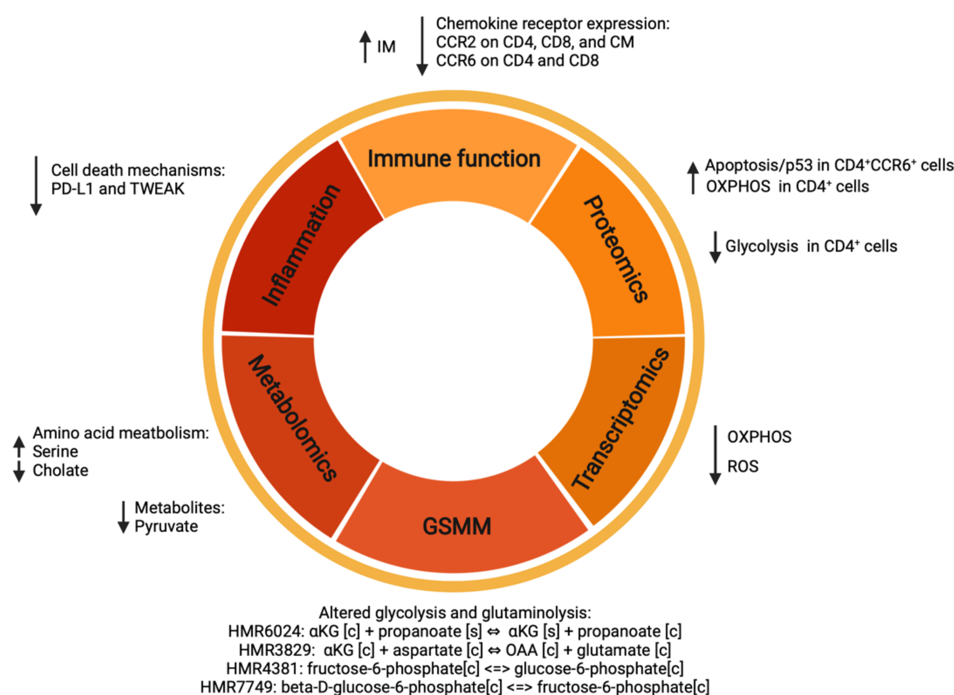

Figure 25: The hallmarks of elite control phenotype in comparison to long-term suppressive therapy. The figure represents the main alterations identified (PLWH$_{EC}$ vs PLWH$_{ART}$) in this thesis.

As proposed in this thesis, the regulation of the HIV-1 provirus and chronic inflammation is tightly interlinked with the immunometabolism of cells. That said, we understand that merely focusing on circulating PBMCs is not sufficient in the context of HIV-1 infection as the main persistence resides in tissue reservoirs. However, secondary alterations of circulating immune subsets, although not all carrying latent HIV-1 themselves, still exhibit deregulated immunometabolism that can contribute to the inflammatory environment during suppressive infection. The increase in inflammatory markers detected here, and in other studies [53-57], may be a result of metabolic dysregulation. It is known that reprogramming of metabolic processes is directly correlated to the activity of the immune system. In **paper III-V**, we showed how long-term successfully treated PLWH have a system level metabolic dysregulation, mainly revolving round OXPHOS and AA metabolism. Specifically, this data indicates a dysregulation of glutaminolysis in PLWH$_{ART}$, possibly contributing to an earlier ageing. However, earlier ageing is most likely not a universal phenomenon affecting all PLWH but rather affecting a subset of infected individuals during suppressive ART. This could be a consequence of late ART initiation and adherence or genetic pre-dispositions. Furthermore, we report that the altered metabolic environment is presumably a consequence of cells from myeloid cell lineages. Therefore, it would be interesting to identify the functional capacity of MNPs during suppressive ART. Specifically, since myeloid cells are contributors to the HIV-1 reservoir, it is possible that an increased CCR5 expression makes CM and some DC populations more susceptible than originally thought [91]. Consequently,

their capacity for tissue residency into areas with low drug penetration could provide compartments where this part of the reservoir is replenished and sustained during therapy.

All the studies included in this thesis touch on the dysregulation of immunometabolism during suppressive HIV-1, but the complete mechanisms are not fully elucidated. Further understanding of how the aberrant metabolism affects immune cell functions, specifically in subpopulations from myeloid cell lineages, are warranted. As myeloid cells are a part of the primary defence in the host, the impact of the immunometabolic dysregulation on secondary innate and adaptive immune responses also need further attention. To understand the direct effect of HIV-1 persistence, studies on metabolic reprogramming in primary latency models would also be beneficial. Moreover, this thesis mainly looked at metabolic profiling of the bulk cell populations. Given that metabolic alterations are plastic, and reversible depending on the condition, another avenue of research would be to look at immunometabolic regulation at a single-cell resolution.

The field of immunometabolic reprogramming during HIV-1 infection is rapidly evolving and the main findings covered in this thesis may contribute to our understanding of important aspects to this exciting new field.

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