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# The role of cellular metabolism in the establishment of HIV-1 reservoir

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**Université Paris Cité**

**École doctorale *Bio Sorbonne Paris Cité ED 562*  
Laboratoire *HIV, inflammation et persistance, Institut Pasteur***

# **THE ROLE OF CELLULAR METABOLISM IN THE ESTABLISHMENT OF HIV-1 RESERVOIR**

Par Amal ELFIDHA

Thèse de doctorat d'Infectiologie

Dirigée par Asier SÁEZ-CIRIÓN

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## **Titre : Le rôle du métabolisme cellulaire dans l'établissement du réservoir VIH-1**

**Résumé :** Grâce à la thérapie antirétrovirale (TAR), il est possible de contrôler la réplication du VIH-1 chez les personnes vivant avec le VIH-1. Néanmoins, la TAR seule n'est pas en mesure d'éradiquer le VIH-1 puisque, sauf dans de rares cas, la propagation du virus reprend en quelques semaines si la TAR est interrompue, même après des décennies de traitement régulier. Des cellules infectées à longue durée de vie, résistantes aux traitements, continuent à contenir des provirus intacts et capables de se répliquer. Ce réservoir est à l'origine du rebond viral rapide qui se manifeste lorsque la TAR est interrompue et sa persistance est un obstacle à la guérison de l'infection par le VIH-1.

Si le VIH-1 cible préférentiellement les T CD4+, ces cellules ne montrent pas une permissivité homogène à l'infection. Il a été démontré que la susceptibilité à l'infection augmente avec la différenciation et l'activation de ces cellules. Certaines études suggèrent que la susceptibilité relative des populations des T CD4+ peut varier au cours de l'infection, avec un impact sur la constitution du réservoir du VIH-1 si la TAR est initiée pendant la primo-infection ou en phase chronique. L'expression relative des facteurs cellulaires de dépendance et de résistance régule la dichotomie entre la permissivité et la résistance des T CD4+ à l'infection par le VIH-1. Des travaux récents de l'équipe d'accueil ainsi que d'autres, ont montré que le métabolisme cellulaire joue un rôle clé dans la permissivité des T CD4+ à l'infection par le VIH-1. Ces résultats ont suscité l'intérêt pour un potentiel thérapeutique de ciblage du métabolisme cellulaire dans la lutte contre l'infection.

Dans cette thèse, nous avons exploré *in vitro* les liens entre les voies métaboliques et l'infection par le VIH-1 et comment certaines cytokines, fortement produites en primo-infection, peuvent moduler le statut métabolique des cellules T CD4+ influençant potentiellement leur susceptibilité à l'infection. Nous avons utilisé des molécules dont les cibles métaboliques sont connues et qui ont déjà été employées dans des études cliniques *in vivo*, pour montrer que le métabolisme du glucose et la synthèse des acides gras sont des voies métaboliques déterminantes pour l'infection des T CD4+ par le VIH-1. De plus, nous avons constaté que certains médicaments réduisaient l'activation des T CD4+ sans avoir d'impact sur l'infection par le VIH-1. Ces résultats renforcent l'idée que le métabolisme influence l'infection par le VIH-1 indépendamment de l'état d'activation des T CD4+. D'autre part, nous avons constaté que les cytokines jouant un rôle important dans l'homéostasie des cellules T (comme l'IL-2, l'IL-15 et l'IL-7) favorisaient la glycolyse et la phosphorylation oxydative, augmentant ainsi la sensibilité des T CD4+ à l'infection. Au contraire, l'IL-21 diminue la respiration mitochondriale et l'infection des T CD4+ par le VIH-1 et l'IL-10 et l'IFN- $\alpha$  ont démontré une capacité à contrer l'augmentation de l'activité métabolique de l'IL-2/IL-7.

Nos recherches ont contribué à la compréhension de l'interaction complexe entre le statut d'activation, le métabolisme des T CD4+ et l'infection. Ce système complexe peut être modulé par des voies métaboliques présentant des accélérateurs ou des freins à l'infection VIH-1, ainsi qu'à la réponse aux signaux des cytokines. Nos études ont également identifié des molécules et des cytokines influençant l'immunométabolisme qui pourraient être utilisées dans des thérapies qui contribuent à la rémission du VIH-1.

**Mots clés :** VIH-1, SIDA, cytokine, métabolisme cellulaire, immunométabolisme, lymphocytes CD4+, IL-2, IL-7, IL-15, IL-21, IL-10, IFN



## **Title : The role of cellular metabolism in the establishment of HIV-1 reservoir**

**Abstract :** While it is possible to control viremia for life in people with HIV thanks to antiretroviral therapy (ART), it is now evident that ART alone cannot eradicate HIV since, barring rare instances, the spread of the virus resumes within a few weeks if ART is discontinued, even after decades of thorough and regular treatment. Pools of long-lived infected cells that are resistant to modern antiretroviral drugs continue to harbor replication-competent HIV-1 for decades; they are the cause of the rapid viral rebound that occurs when therapy is interrupted.

Although HIV-1 preferentially targets CD4<sup>+</sup> T cells, these cells do not show homogenous permissibility to infection. Notably susceptibility to infection has been shown to increase with differentiation and activation. Some studies suggest that the relative susceptibility of CD4<sup>+</sup> T cell subsets may vary during primary and chronic infection, affecting the seeding of the HIV-1 reservoir when ART is initiated. The relative expression of dependence and resistance cellular factors regulate the dichotomy between permissibility and resistance of CD4<sup>+</sup> T cells to HIV-1 infection. Recent work by the host team and others has shown that cellular metabolism plays a key role in the permissibility of CD4<sup>+</sup> T cells to HIV-1 infection. These findings have sparked the interest of the therapeutic potential of targeting cell metabolism to tackle HIV-1 infection. In this thesis we have further explored in vitro the links between cell metabolic pathways and HIV-1 infection, and how some cytokines that are strongly produced early in response to HIV-1 infection can enhance or decrease the metabolic status of CD4<sup>+</sup> T cells potentially influencing their HIV-1 susceptibility. We used drugs with known cell metabolic targets and previously employed in clinical studies in vivo to show that glucose metabolism and fatty acid synthesis are decisive cell metabolic pathways affecting HIV-1 infection of CD4<sup>+</sup> T cells. Moreover, we found that some metabolic drugs reduced CD4<sup>+</sup> T cell activation without affecting HIV-1 infection. These results reinforce the idea that cell metabolism influences HIV-1 infection independently of the activation status of CD4<sup>+</sup> T cells. On the other hand, we found that cytokines with important role on T cell homeostasis (such as IL-2, IL-15 and particularly IL-7) promoted glycolysis and oxidative phosphorylation, enhancing HIV-1 susceptibility of CD4<sup>+</sup> T cells to infection. In contrast, IL-21 decreased mitochondrial respiration and HIV-1 infection of CD4<sup>+</sup> T cells, IL-10, and IFN- $\alpha$  were able to counteract the metabolic enhancing effects of IL-2/IL-7.

Our research has contributed to the understanding of the complex interaction between T cell activation, T cell metabolism and HIV-1 infection, composed of parallel pathways with accelerators and breaks to infection, and the response to cytokine cues. Our studies have also identified small molecules and cytokines influencing immunometabolism with potential to be incorporated into therapeutic strategies aiming HIV-1 remission.

**Keywords :** HIV-1, AIDS, cytokine, cellular metabolism, immunometabolism, CD4<sup>+</sup> T cells, T lymphocytes, IL-2, IL-7, IL-15, IL-21, IL-10, IFN

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*In Tunisian dialect: Nochker ommi, Afifa, w baba, Lotfi, el ghalyin, w khweti Majdi w Wael. Barcha ness ykoulouli elli ena kwiya w choujaà khaterni kararet nebaàd ala ayalti w s'habi bech nkawan moustakabli w nebni hyat jdida. Ama mech barcha ness ykhamamou al chajaà elli ommi w baba warouha. Taàddou ochahra w snin baàd ala ayalti, taadhabet fihom ena ama zeda ommi w baba elli lazemhom yekeblou el hal elli mech bech ynajmou ychoufouni dima. Yaàz alia le fait elli omerhom mahassassouni bedhanab ki manajemtech nji nchoufhom. Mama, Papa merci beaucoup ala kolchay wafartouhouli, menghirkom entou makontech najam nhakkak ahlemi. Merci beaucoup khater même ken tbadalet alikom w baàd saàt manetwafekech maakom ala barcha hayat, dima araftou kifech tekfou maaya w t'hebbouni à distance w t'hassesouni elli manich wahdi. Merci beaucoup khaterkom wthaktou fiya, chajaàtouni w awantouni. Ena fakhoura elli nkoun benetkom w nchallah nrajaàkom dima fiers biya. W nouadkom elli snin elli jeya bech tchoufouni akthar barcha. Wael et Majdi, j'espère elli hatta entouma fakhourin biya w j'espère elli najjam nkoun bjanebkom w nchoufkom farhanin w t'hakkakou ahlemkom zeda. Rabi ykhalikom liya. Nhebbkom barcha.*

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"Courage is going from failure to failure without losing enthusiasm." — Winston Churchill  
"Be kind, for everyone you meet is fighting a battle you know nothing about." — Ian Maclaren

# ABBREVIATION LIST

ACC	Acetyl-CoA Carboxylase	NF-AT	Nuclear factor of activated T cells
AHI	Acute HIV infection	NF- $\kappa$ B	Nuclear factor-kappa B
AICD	Activation-induced cell death	NK	Natural killer
AIDS	Acquired immunodeficiency disease syndrome	NKT	Natural killer T
Akt	Protein kinase B	NLRP3	NOD-like receptor family, pyrin domain containing 3
AMPK	AMP-activated protein kinase	NNRTI	Non-nucleoside reverse transcription inhibitors
APC	Antigen-presenting cell	NPC	Nuclear pore complex
APOBEC3	Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3	NRTI	Nucleoside reverse transcription inhibitors
ASCT2	cellular receptor for neutral AAs such as glutamine and leucine	p56lck	Lymphocyte-specific protein tyrosine kinase 56
ATP	Adenosine triphosphate	PD-1	Programmed cell death protein 1
CD	Cluster of differentiation	pDCs	Plasmacytoid DCs
CD4+ T	CD4+ T lymphocytes	PI3K	Phosphoinositide 3-kinase or phosphatidylinositol 3-phosphate kinase
CPT1	Carnitine palmitoyl transferase I	PKC	Protein kinase C
CTL	Cytotoxic	PLWH	People living with HIV
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4	Pol	Polymerase
CXCL10	C-X-C motif chemokine ligand 10	PPAR	Peroxisome proliferator-activated receptors
CXCR4	C-X-C Motif Chemokine Receptor 4	PPP	Pentose phosphate pathway
CypA	Cyclophilin A	PR	Protease
DC-SIGN	Dendritic cell-specific ICAM-grabbing non-integrin	PTEN	Phosphatase and tensin homologue deleted on chromosome 10
dNTPs	Deoxynucleotide Triphosphates	Raf	Rapidly accelerated fibrosarcoma kinase
EC	Elite controllers	Rev	RNA splicing-regulator
eIF4A	Eukaryotic initiation factor-4A	ROS	Reactive oxygen species
Env	HIV-1 Enveloppe	RT	Reverse transcriptase
ERK	Extracellular signal-regulated kinase	SAMHD1	Sterile A Motif and Histidine Aspartate domain-containing protein 1
ERK	Extracellular-signal-regulated kinase	SERINC5	Serine incorporator proteins
FAO	Fatty acid $\beta$ -oxidation	SH2	SRC homology-2
FASN	Fatty Acid Synthase	SIV	Simian immunodeficiency virus
Gag	Group specific antigen	SLC	Solute carrier
GDH	Glutamate dehydrogenase	SNAT1	Sodium-coupled neutral AA transporter 1
GLS	Glutaminase	SNP	Single nucleotide polymorphism
GLUT1	Glucose transporter 1	SOCS	Suppressors of cytokine signaling
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor	SREBP	Sterol regulatory element binding protein
Gp	Glycoprotein	STAT	Signal transducer and activator of transcription
HDFs	HIV dependency factors	Tat	Transactivator protein
HIC	HIV Controllers	T-bet	T-box expressed in T cells
HIF-1 $\alpha$	Hypoxia-Inducible Factor-1 $\alpha$	TCA	Tricarboxylic acid
HK	Hexokinase	TCR	T cell receptor
IDO	Indoleamine 2,3-dioxygenase	T <sub>N</sub>	CD4+ T with a naïve phenotype
IFITMs	INF-induced transmembrane proteins	T <sub>SCM</sub>	T stem cell memory
IFN	Interferon	T <sub>CM</sub>	T central memory
IL	Interleukin	T <sub>TM</sub>	T transitional memory
IRF	Interferon regulatory factor	T <sub>MM</sub>	T migratory memory
Jak	Janus kinases	T <sub>RM</sub>	T tissue resident memory
LDH	Lactate dehydrogenase	T <sub>EM</sub>	T effector memory
LEDGF	Lens epithelium-derived growth factor	T <sub>TD</sub>	T terminally differentiated
LPA	Lysophosphatidic acid	T <sub>REG</sub>	T regulatory
LPS	Lipopolysaccharide	T <sub>FH</sub>	Follicular T helper cells
MAPK	Mitogen-activated protein kinase	T <sub>H</sub>	T helper
Mcl-1	Myeloid cell leukemia sequence 1	TLR	Toll-like receptor
MEK	Mitogen-activated protein kinase	TNF- $\alpha$	Tumor necrosis factor $\alpha$
mTOR	Mammalian target of rapamycin	TRIM5 $\alpha$	Tripartite motif (TRIM) protein 5 $\alpha$
mTORC1	mTOR Complex 1	TYK2	Tyrosine Kinase-2
mTORC2	mTOR Complex 2	Vif	Viral infectivity factor
MyD88	Myeloid differentiation primary response 88	Vpr	Virus protein r
NAD <sup>+</sup>	Nicotinamide adenine dinucleotide	Vpu	Virus protein U
NADH	Nicotinamide adenine dinucleotide + hydrogen	Vpx	Viral protein X
NADPH	Nicotinamide adenine dinucleotide phosphate	$\alpha$ -KG	$\alpha$ -ketoglutarate
Nef	Negative regulating factor		

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# INTRODUCTION

## Overview of the AIDS pandemic

In 2021, around 38.4 million individuals worldwide were living with human immunodeficiency virus (HIV), with 1.5 million new infections occurring that year<sup>1</sup>. The global rate of new infections has held steady at approximately 2 million new infections per year, adding continuously to the number of people requiring lifelong treatment. Since the early 1980s, major advances have been made with treatment for people living with HIV (a community hereby referred to as PLWH). Contemporary antiretroviral therapy (ART) has transformed HIV infection from a life-threatening infection that causes the acquired immunodeficiency disease syndrome (AIDS) to a manageable chronic health condition, enabling PLWH with access to ART to lead healthy and long lives.

Despite significant advances, there are several obstacles to ART implementation. ART can effectively block HIV replication and prevent or reverse AIDS in PLWH. However, ART does not eliminate cells harboring HIV integrated viral genome, and viremia re-emerges within weeks after discontinuing therapy<sup>2,3</sup>, making ART a life-long treatment. Out of the 38 million individuals living with HIV, only 28.7 million people had access to ART. Many PLWH renounce HIV testing and treatment because of the associated stigma, leading to suboptimal care<sup>4</sup>. Factors such as costs and misinformation (or lack of information), are also major obstacles for universal ART access. In addition, many other people are unable to achieve long-term viral suppression despite receiving ART<sup>5</sup>.

Whereas the life expectancy for PLWH has gradually increased, non-AIDS morbidity has grown more prevalent. With progressive ART coverage, care has transitioned away from immunodeficiency-related opportunistic infections or AIDS-defining cancers and towards ART-related issues (such as toxicities, medication-drug interactions, or antiretroviral drug resistance) and, more recently, to different non-AIDS disorders<sup>6-8</sup>. Such comorbidities may be the result of long-term ART toxicities, inflammation caused by HIV infection, and aging<sup>9-15</sup>. Furthermore, problems with toxicity and intolerance often result in suboptimal adherence to the treatment regimen<sup>16</sup>. However, adherence to ART is crucial for obtaining sufficient suppression of HIV replication<sup>17</sup>, which is essential to reduce the formation of drug resistance<sup>18</sup>, another major challenge in ART-based strategies. Non-adherence to ART can be attributed to factors such as daily intake and adverse effects of the treatment.

Considering the requirement for lifelong HIV treatment with ART, as well as the related costs, side effects, and stigma, there is still a need for a global scientific effort to end the HIV pandemic. Reports from five persons who may have achieved HIV cure after receiving allogeneic stem cell transplant, as well as examples of long-term HIV remission after ART discontinuation, have greatly motivated researchers to reorient their efforts on finding an HIV cure<sup>19,20</sup>.



# HIV replication and antiretroviral therapy

## HIV biology

HIV-1 was identified in 1983 as the causal agent of AIDS<sup>21</sup>. HIV-1 belongs to the lentivirus genus of the *retroviridae* family. HIV can be divided into two major types: type 1 HIV (HIV-1, referred to as HIV in this manuscript) and type 2 HIV (HIV-2).

HIV genome consists of a double copy of positive, single-stranded RNA molecules of about ~9200 to 9600 nucleotides that contain nine genes and encode 15 viral proteins. Briefly, this genomic organization consists of an arrangement of the genes *gag*, *pol* and *env* together with six extra accessory genes that are flanked by respective 5' and 3' untranslated regions (**Figure 1A**). The three structural genes *gag*, *pol* and *env*, which are commonly encoded by all retroviruses, are synthesized as polyproteins. Gag polyprotein (group specific antigen, also called p55) produces proteins for virion interior; proteins of the outer core membrane (MA, p17), the capsid protein (CA, p24), the nucleocapsid (NC, p7) and a smaller, nucleic acid-stabilizing protein. Pol (polymerase) polyprotein codes for the viral enzymes: enzymes protease (PR, p12), reverse transcriptase (RT, p51) and RNase H (p15) or reverse transcription plus RNase H (together p66) and integrase (IN, p32). Finally, the *env* polyprotein (envelope, gp160) produces the surface glycoproteins for the virion: gp120 (surface protein, SU) and gp41 (transmembrane protein, TM). HIV genome also codes for a number of auxiliary proteins in addition to the structural proteins: Nef (negative regulating factor), Vif (viral infectivity factor), Vpr (virus protein r), and Vpu (virus protein unique) which all regulate the viral replication, virus budding, and pathogenesis, whereas Tat (transactivator protein) and Rev (RNA splicing-regulator) are required for the initiation of HIV replication.

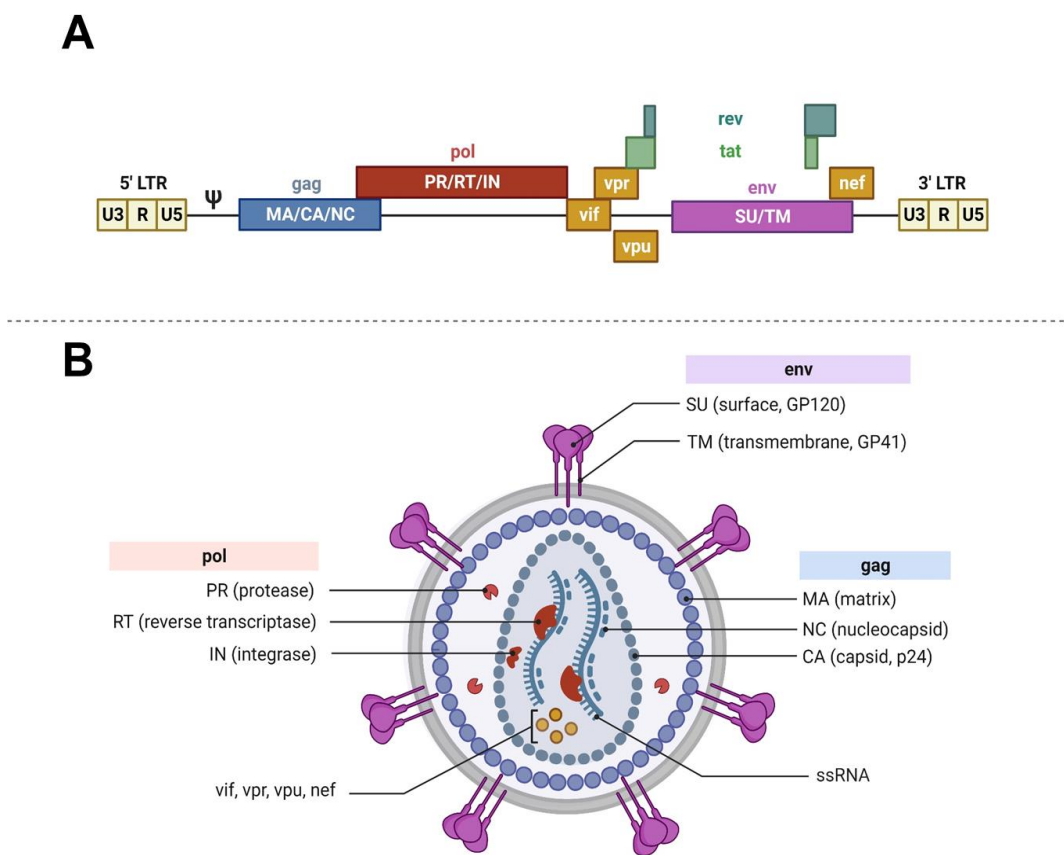
In a mature infectious HIV virion, the viral genome is packaged as a condensed ribonucleoprotein complex with nucleocapsid, integrase, and other associated viral and cellular proteins, within a conical capsid core comprised of the viral capsid protein p24 (**Figure 1B**). The tapered pole of the capsid is attached to the outer capsid membrane, which is formed by the matrix protein p17. The viral envelope covers this outer capsid and is composed of a lipid bilayer and, the envelope proteins gp120 and gp41. The viral particle, as well as the lipid bilayer which is derived from the host cell, contain cellular proteins that are packaged during the assembly of the virion. HIV replication cycle is comprised of several distinguishable steps, that were estimated to take approximately 16 to 24 hours for one round of infection *in vitro* in CD4+ T cells<sup>22</sup>. More than 30 antiretroviral molecules, targeting each of these replication steps, have been approved for treatment of HIV infection<sup>23</sup>.

### Tropism

HIV enters the body through the exchange of bodily fluids, and it infects mainly CD4+ T cells, macrophages and, to some extent, microglial cells and dendritic cells (DCs). This tropism is determined at the level of viral entry by the usage of the cluster of differentiation 4 (CD4) proteins as a main receptor and the use of co-receptors which are strain and target cell specific. The binding affinity between gp120 and C-C chemokines receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) to a large extent determines the cell tropism of a particular viral isolate. HIV strains that use the signaling receptor CCR5 as a co-receptor in addition to CD4, are referred to as R5. Viruses that primarily use CXCR4 are denoted X4. R5X4 or “dual-tropic” strains can enter cells via either CCR5 and CXCR4. R5 strains can infect macrophages

and CD4+ T cells, while X4 strains can infect only CD4+ T lymphocytes, which express CXCR4 co-receptor.

During the primary or early, asymptomatic stage of HIV infection, only R5 viruses are detected in PLWH and R5 viruses are largely responsible for person-to-person transmissions<sup>24</sup>. Viruses that employ CXCR4 for entry arise during the late stage of HIV infection and become predominant thereafter. The switch from R5 to X4 tropism in people is often associated with dramatic decline in CD4+ T cells count and the onset of AIDS-defining symptoms in the absence of therapy. The virus life cycle could be divided into eight steps: entry, reverse transcription, uncoating and nuclear entry, integration, transcription, translation and assembly, followed by viral budding (**Figure 2**).

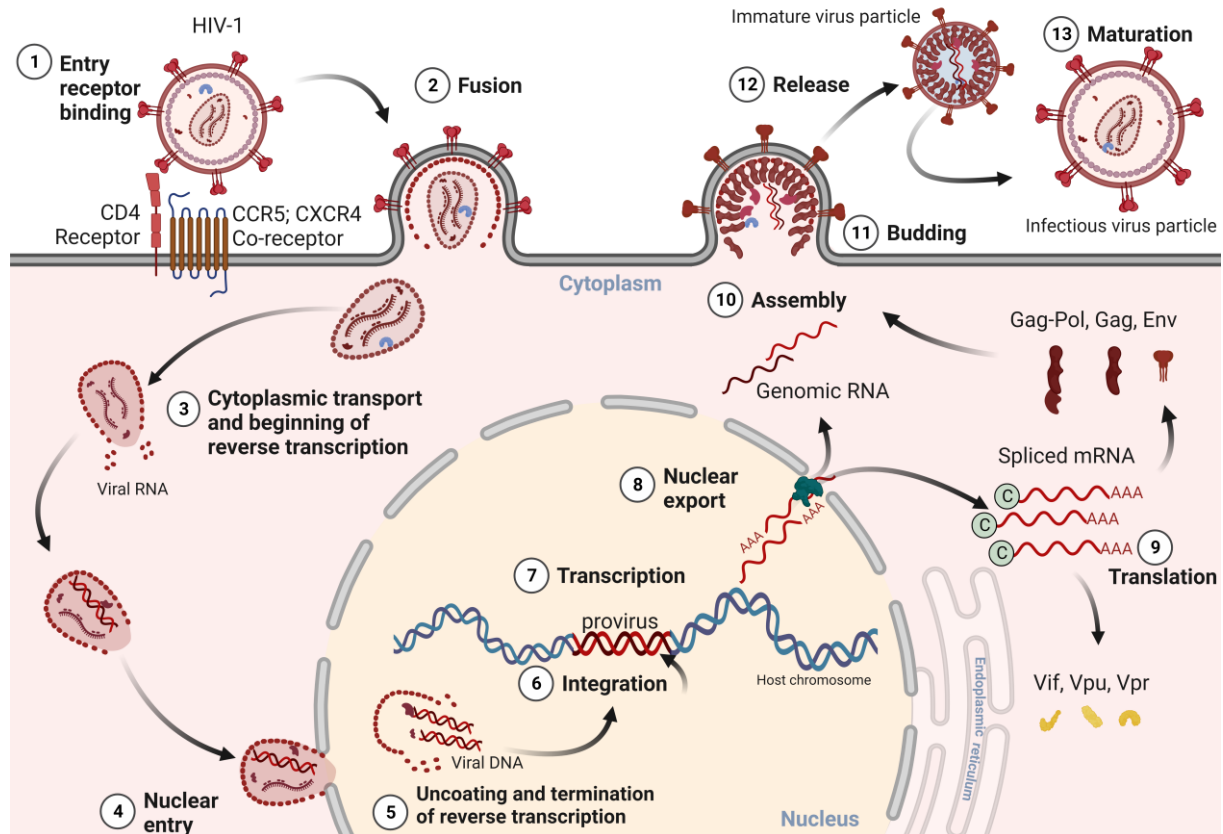


**Figure 1. Schematic representation of HIV-1 genome and virion structure.**

(A) Schematic overview of the organization of the HIV-1 genome encompassing open reading frames coding for different structural, regulatory, and accessory proteins. The dimeric, linear gRNA is ~9 kb long and is flanked by 5'- and 3'-long terminal repeats (LTRs) that contain the viral promoter and sequences required for reverse transcription, integration, and gene expression. The LTRs are distinguished into cis-acting regulatory elements, namely the U3, R, and U5 regions, followed by the packaging signal Psi ( $\Psi$ ). Gag encodes the structural proteins matrix (MA), capsid (CA), and nucleocapsid (NC) forming the viral core. Pol codes for the viral enzymes protease (PR), reverse transcriptase (RT), and integrase (IN). The Pol gene is followed by two regulatory genes, rev and tat, and three accessory genes, vif, vpr, and vpu. Env encodes the viral envelope glycoproteins, the surface unit (SU) gp120, and the transmembrane unit (TU) gp41. Env is followed by another accessory gene nef. (B) Schematic view of the HIV particle. The mature enveloped virion has a spherical shape and is enveloped by a lipid bilayer membrane derived from the host cell containing 7–35 envelope glycoproteins trimers. The inner layer of the membrane anchors the Gag-derived MA proteins and also harbors Vpr and PR. The capsid is found within the center of the virion and contains the two copies of gRNA, RT, and IN. The gRNA is stabilized by the NC proteins.

## Entry

The virus replication cycle begins with entry of the virus into the host cell. This stage comprises three steps: attachment, binding and fusion. Gp120 on the virion surface recognizes and binds to the CD4 receptor on the cell surface. CD4 binding induces conformational changes in the Env trimer that allow the interaction of gp120 with either the CXCR4 or CCR5 co-receptors. Co-receptor interaction triggers additional conformational change of the gp120-gp41 complex that results in the release of fusion peptide and fusion of the viral-cell membrane.



**Figure 2. Schematic overview of HIV-1 replication cycle.**

(1) HIV-1 infection begins with the attachment of the envelope glycoprotein gp120-SU to the primary CD4 receptor and the chemokine co-receptor CCR5 on the host cell surface. Viral variants that utilize the CXCR4 co-receptor appear later during infection. (2) This binding results in the viral and cellular membranes fusing and the viral particle entering the cell, the viral capsid is then released into the cytoplasm. (3) Partial capsid uncoating facilitates reverse transcription. Reverse transcriptase initiates the synthesis of viral DNA, while the capsid is transported to the nucleus. (4) The capsid docks to the nuclear pore complex and passes through pores into the nucleus. Uncoating is the process by which the viral cDNA genome is released from the capsid. It is a multistep process that is closely associated with reverse transcription. (5) The capsid uncoats during nuclear cell entry, reverse transcription is completed inside the nucleus and the pre-integration complex (PIC) is formed. (6) Viral cDNA is covalently integrated into the host genome by integrase, preferentially at transcriptionally active sites. The integrated viral genome is referred to as a provirus. (7) The provirus can be transcribed to the point where it reaches *tat* expression and enters the replicative phase. Alternatively, it can be methylated by host factors, and transcriptional silencing induces viral latency. Nuclear signals can abrogate the transcriptional silencing of the provirus, initiating the viral replication cycle. (8) Proximal transcription factors that interact with the U3 promoter or *tat* activate provirus transcription. mRNA transcripts and genomic RNA (gRNA) are exported to the cytoplasm. (9) Proviral transcription, mediated by host RNA polymerase II (RNA Pol II) and positive transcription elongation factor b (P-TEFb), generates viral mRNAs of varying sizes, the larger of which require energy-dependent export via the host's cellular nuclear export factor Exportin 1, to exit the nucleus. (10) Two viral gRNAs, Gag, Pol, Env, and Vpr assemble to nascent HIV-1 particles at the cell membrane. (11) Immature HIV-1 particles bud at the cell membrane. (12) Immature HIV-1 particles are released from the host cell to the extracellular milieu. (13) Upon the release of the viral particle, the protease is activated and cleaves the polyproteins GAG and GAG-POL into individual chains that self-assemble in the virion to form a conical capsid.

### **Reverse transcription**

Upon fusing with the cell membrane, HIV particles containing the viral RNA can enter into the cell's cytoplasm. Exposure of the viral ribonucleoprotein complex to non-limiting deoxyribonucleotides triggers the viral reverse transcriptase and the single-stranded viral RNAs are reverse-transcribed into linear double-stranded DNAs. Reverse transcription is characteristic, although not unique, to all retroviruses and is a very complex series of events.

### **Uncoating and nuclear entry**

HIV uncoating refers to the disintegration of the viral capsid prior to the import of the viral DNA into the nucleus of the cell. Early research revealed that viral entrance might be followed promptly by uncoating. However, more recent results indicate that capsid stability, which may last for several hours, is essential for HIV infectivity<sup>25-28</sup>. Intensive research is being done to better understand the precise time of uncoating. Lentiviruses are distinguished from other retroviruses by the ability to infect non-dividing terminally differentiated cells, such as macrophages and this implies that the viral genome must be transported through an intact nuclear membrane. The pre-integration complex (PIC) is too large to allow for passive diffusion and has to be actively imported into the nucleus. It was believed that the matrix protein, Vpr and integrase are implicated in the nuclear transport of the PIC. Recent studies, however, indicate that none of these viral proteins is necessary for the infection of non-dividing cells and that the capsid protein may be important for nuclear entry and intra-nuclear trafficking as well as for cytoplasmic trafficking, reverse transcription, shielding of viral nucleic acid from the innate immune system, although the underlying mechanisms are still not completely known. Viral proteins may mediate nuclear import directly or via the recruitment of the host's proteins.

### **Integration**

The newly synthesized viral DNA is then translocated across the nuclear pore as part of the high-molecular-weight PIC. The viral genome must be inserted into that of the host cell for gene expression and productive infection. The integration process is catalyzed by the viral integrase. Once the viral DNA is inserted into the cellular DNA; the cell becomes infected for the remainder of its life span. The proviral DNA is reproduced alongside the host DNA as a component of the chromosome of the host cell. As a result, the spread of infection can be done by infection of new cells or by the proliferation of cells that already contain the proviral DNA. The integrated viral genome, also known as a provirus, may remain latent in some long-lived cells for many years. These infected long-lived cells constitute the cellular reservoir and pose a major obstacle to the eradication of HIV. Discontinuation of ART, even after many years of therapy, causes the proviruses to become active and produce infectious new HIV particles.

### **Transcription**

In productively infected cells, the integrated HIV provirus serves as a template for the transcription of both viral proteins messengers and genomic RNA by the cellular RNA Polymerase II. Proviral transcription is initiated by a viral promoter but viral gene expression is cellular transcription factors like nuclear factor-kappa B NF- $\kappa$ B and nuclear factor of activated T cells (NF-AT) are necessary for viral gene expression. The viral protein Tat boosts viral transcription, RNA elongation and allows for efficient synthesis of the full-length HIV transcripts and several mRNAs generated by alternative splicing. Rev interacts with the viral

transcripts and allows for transport of unspliced and partially spliced mRNAs from the nucleus to the cytoplasm.

### **Translation and Assembly**

Synthesis of Tat and Rev leads to the production of full-length unspliced mRNA that expresses the Gag and Gag-Pol precursors, which are then processed to major structural and enzymatic proteins. In parallel, single-spliced viral RNAs are used for the synthesis of Vif, Vpr, Vpu, and Env proteins. After their translation, viral structural and enzymatic proteins travel to the plasma membrane, where immature virions assemble in cholesterol-rich lipid rafts. The process of viral assembly is a complex and highly ordered process. Broadly sketched, the Gag and Gag-Pol precursors multimerize via interactions between Gag proteins. Additionally, the matrix domain of both precursors is N-terminally myristoylated, causing them to concentrate in lipid rafts at the inner leaflet of the plasma membrane. Similarly, viral Env glycoproteins are attracted to these assembly platforms through interactions with the matrix protein. Finally, interactions between the zinc fingers located in the NC domain of Gag and the stem loops packaging signal of the viral genome enable the recruitment of two RNA copies to this assembly complex. Moreover, the viral Vif protein, as well as some cellular factors, are also recruited to the sites of virion assembly and incorporated into viral particles. The accumulation of viral proteins and RNA at the plasma membrane causes the curvature of this latter and the formation of membrane-coated spherical particles.

### **Budding**

The release of progeny virions from the infected cells is called budding. Ubiquitination of carboxy-terminus of the p6 part of Gag creates high affinity binding sites that recruit components of multivesicular bodies, such as tumor-susceptibility gene 101 (TSG101) and vacuolar protein sorting 4 (VPS4) to assist in the final stages of budding. These components allow newly formed HIV particles to be released.

### **Maturation**

HIV virions are released in an immature and noninfectious form, with a thick coating of radially packed Gag and Gag-Pol precursors. During or shortly after budding, the viral protease becomes activated and cleaves Gag and Gag-Pol precursors. Processing of Gag and Gag-Pol yields mature HIV particles. As a result, the configuration of the viral proteins is re-organized to produce the signature electron-dense conical inner core and render the virus contagious. Some accessory viral proteins such as Vpr and Nef, as well as cellular components are incorporated into these particles.

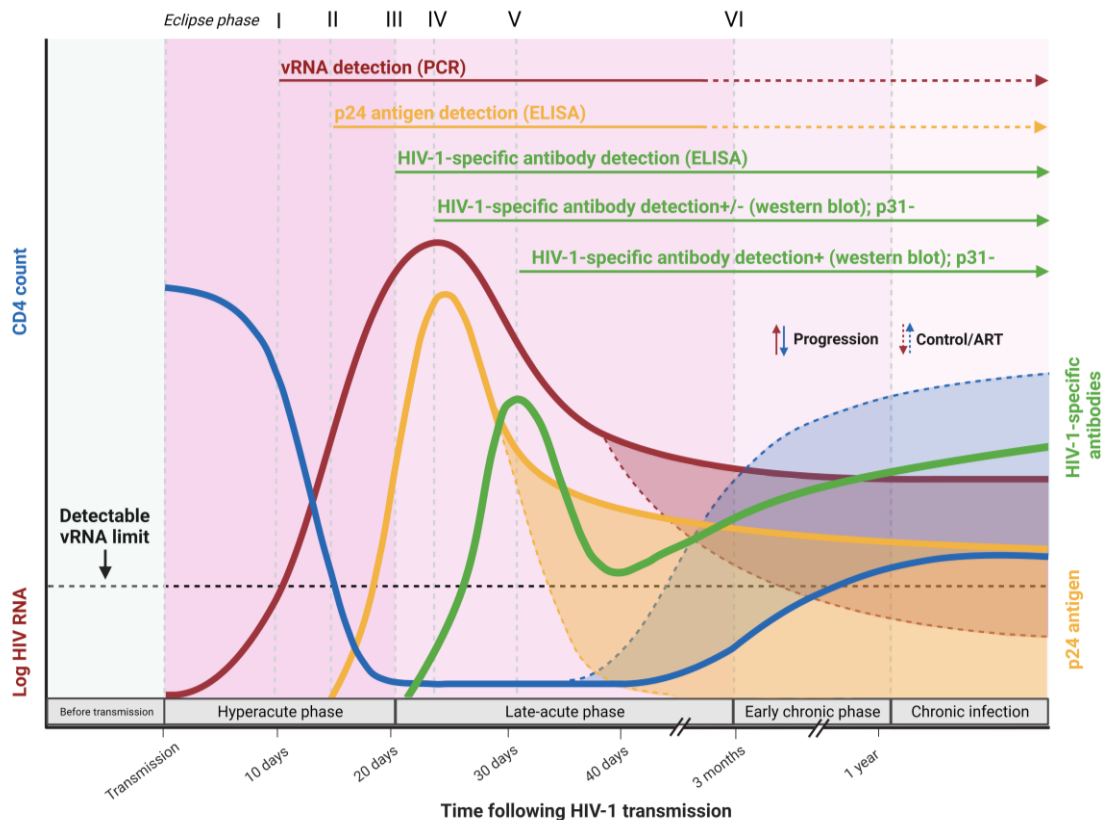
## **Natural history of HIV infection**

Virological and immunological events during acute HIV infection (AHI) are linked to disease etiology. The virus replicates at the infection site and in local lymphoid tissues for the first 10 days of HIV infection (eclipse phase). Subsequent stages of AHI are defined by the detection of viral RNA only (Fiebig stage I), viral RNA and p24 antigen (Fiebig stage II), and production of HIV-specific antibodies (Fiebig stages III-V) (**Figure 3**)<sup>29</sup>.

### Eclipse phase

The initial phase, also called “eclipse phase” lasts for 10 days and defines the period in which the virus is not detectable in the plasma. In SIV-challenged rhesus macaques, a model closely resembling HIV-1 infection in humans, a robust systemic pro-inflammatory response takes place within the first 1 to 3 days of this phase, with the induction of innate immune activity, including components of the inflammasome, which is hypothesized to contribute to the suppression of the host cell’s restriction to infection<sup>30,31</sup>. By the end of the “eclipse phase”, HIV gains access to the draining lymph nodes, through the migration of DCs, where its replication significantly amplifies. The abundance of activated T cells, the capture of virions on the extracellular surface of follicular DCs, and the excessive production of pro-inflammatory cytokines like interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, or tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) that strongly support HIV replication make the lymphoid tissue an ideal environment for virus replication and spread<sup>32,33</sup>.

Plasmacytoid DCs (pDCs) are among the earliest first-responders during acute viral infections. *In vitro*, pDCs become activated by the binding of viral envelope proteins to CD4 followed by virion endocytosis and by the triggering of Toll-like receptor 7 (TLR7) by viral RNA, which induces a rapid and robust production of type I interferons (IFNs).



**Figure 3. Representative dynamics of plasma viral load, peripheral CD4+ T cell counts, p24 antigen and antibody detection throughout the course of HIV infection.**

Individuals that develop control of HIV without ART can maintain low chronic viral set point and reestablish pre-infection CD4+ T cell levels (elite or viremic controllers). Individuals and animals can also maintain high chronic viral set point and recover CD4+ T cell counts (long-term non-progressors and natural hosts, respectively).

These cytokines in turn have a complex and pleiotropic effect on all components of the immune system, particularly T cells. pDCs also upregulate activation, migration markers and recruit other immune cells through the production of chemokines such as CCL4.

A recent study of people during hyper-acute HIV infection has shown an initial high capacity for IFN production, however, within one week, pDCs in the blood showed a transient decline in the ability to produce IFN- $\alpha$  after *in vitro* stimulation<sup>34</sup>. pDCs also displayed decreased expression of integrin  $\beta$ 7, a cell membrane-bound trafficking protein, indicating that these cells were unable to move to mucosal sites where the virus mostly replicates. Moreover, HIV-exposed pDCs also generate indoleamine 2,3-dioxygenase (IDO), which stimulates CD4+ T cells to differentiate into regulatory T ( $T_{REG}$ ) cells that could suppress HIV-specific immunological responses<sup>35</sup>. During this "eclipse phase", no effector components of the immune response to HIV appear to be operational to stop the initial spreading of infection.

### **Hyper acute phase**

Amplification of the infection in the draining lymph nodes causes HIV viral RNA to become detectable in the plasma (Fiebig Stage I), and infection spreads to other anatomic compartments like the gut-associated lymphoid tissues (GALTs), which are primarily populated of CD4+ CCR5+ memory T cells. In humans, the onset of plasma viremia is marked by elevated levels of IFN- $\alpha$ , CXCL10 (previously known as IP-10), IL-8, IL-10, IL-12, and CCL2 in the periphery. High levels of certain cytokines (such as IFN- $\alpha$  and IL-15) during the acute infection correlate with peak viremia and have been suggested to contribute to long-term immunodeficiency<sup>36</sup>. The first immunomodulators in the acute cytokine storm (IFN- $\alpha$  and IL-15) are centrally involved in rapidly arming and activating natural killer (NK) cells following infection. NK cells expand rapidly, activate and express cytolytic gene modules within one week post-onset of viremia. NK cells can control HIV replication through cytolysis of virally infected cells and the production of antiviral cytokines and chemokines. However, interactions between NK and DC cells may result in lysis of immature DCs, through the secretion of IFN- $\gamma$ , and preservation of mature DCs that induce inhibitory signals to NK cells by the interaction of upregulated human leukocyte antigen (HLA) molecules and NK receptors. These processes have been reported to be defective during HIV infection as Nef alter the expression of HLA molecules in DCs<sup>37</sup>. In addition, NK cells can modulate DCs, lymphocytes T and B to kill infected cells and provide adequate and potent adaptive immune responses. Higher numbers of NK cells in lymph nodes and B follicles, the control of viral replication in these compartments and the absence of infected T follicular T helper cells ( $T_{FH}$ ) cells are some of the main differences between pathogenic (rhesus macaques, which progress to AIDS) and non-pathogenic (african green monkeys, which do not progress to disease despite high viremia) SIV infections<sup>38</sup>.

In humans, resistance to HIV infection and a delayed progression of AIDS have both been linked to increased NK cell activity as seen by enhanced cytotoxic capacity, IFN and chemokine production<sup>39</sup>. Furthermore, studies on killer cell immunoglobulin-like receptor (KIR) and HLA genotypes of PLWH show that some KIR-HLA interactions are associated with disease susceptibility and progression<sup>40</sup>. For instance, Bw4-80I genotype in combination with KIR3DS1/3DL1 is associated with low-level viraemia and slow disease progression. The mechanisms underlying this better control are not completely clear, but the interactions between KIR3DS1/KIR3DL1 and their Bw4 ligands may determine the expansion of specific subpopulations of NK cells or the licensing of NK cells with increased responsiveness. Recent

studies suggest that changes in the peptides bound by HLA molecules may critically impact the way KIRs are stimulated by the HLA class I/peptide complex. Along these lines, compelling evidence of the impact of NK cells on virus control in the context of a particular KIR background comes from the observation that HIV evolves to evade NK-cell-mediated immune pressure by selecting for sequence variants that specifically affect KIR binding to HLA class I ligands.

Similar to DCs, because of their ability to bind the virus via the complement receptor CD21 (also known as CR2), B cells may also contribute to the early spread of infection. While B cells are not reduced in the early stages of HIV infection, B cell responses are compromised because other cell types vital to the formation of germinal centers (such as T<sub>FH</sub>) are destroyed<sup>41</sup>.

Monocytes and macrophages were shown to upregulate genes encoding many pathogen recognition receptors, restriction factors as well as cytokine production, after onset of plasma viremia<sup>42</sup>.

HIV-specific CD4<sup>+</sup> T cell responses increase during acute HIV infection, but they quickly diminish, as HIV-specific CD4<sup>+</sup> T cells are most vulnerable to HIV infection<sup>43,44</sup>. HIV-specific CD4<sup>+</sup> T cells have a skewed functional profile, which is linked to a decreased IL-2 production and proliferation potential<sup>45</sup>. It was later discovered that the expression of the co-inhibitory molecules programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) was linked to this functional impairment<sup>46,47</sup>. Several studies have assessed HIV-specific CD8<sup>+</sup> T cell responses during early HIV infection, prior to the detection of antibodies. CD8<sup>+</sup> T cell response is detected as viremia approaches its peak and the peak of CD8<sup>+</sup> response coincides with the decrease of viremia levels<sup>48</sup>. The preservation of a homogenous founder virus without signs of immune-driven selection of viral escape mutations suggests that the first CD8<sup>+</sup> T cell response appears to have little to no impact on HIV replication<sup>49</sup>. However, at the time of peak CD8<sup>+</sup> T cell response and decline of viremia, escape mutant viruses start emerging as a result of selected mutations in the epitopes recognized by CD8<sup>+</sup> T cells. Early T cell responses are frequently Env and Nef-specific. Specific responses to other viral proteins, like as the conserved Gag p24 and Pol proteins, tend to emerge during subsequent rounds of T cell responses<sup>50</sup>.

The first CD8<sup>+</sup> T cells responses remain strong despite of the impaired function of CD4<sup>+</sup> T cells. However, their progression to long-term memory cells could be impaired as a consequence of suboptimal help from CD4<sup>+</sup> T cells. In the absence of ART, the levels of viremia begin to drop around week 4 after infection and eventually approach the "virologic" set point, which typically occurs 12 to 16 weeks after infection and signifies the shift from the acute to the chronic phase of the infection. A portion of the immune response's role in the fall of viremia is mediated by HIV-specific cytotoxic CD8<sup>+</sup> T cells, which have the potential to kill HIV-infected CD4<sup>+</sup> T cells. In addition, a significant factor in the decline of viremia may be the absence of cell targets for HIV, as a result of the significant reduction in CD4<sup>+</sup> T cells caused by the direct cytotoxic action of the virus, CD8<sup>+</sup> T cell killing, and activation-induced cell death. The virus expands exponentially, leading to a significant loss of CD4<sup>+</sup> T cells and, in most cases, an immune system that is overworked and unable to catch up. It is thought that the establishment of the latent reservoir, occurs before or very early during the Fiebig Stage I, prior to the development of operational immune responses<sup>51</sup>.



### Acute phase

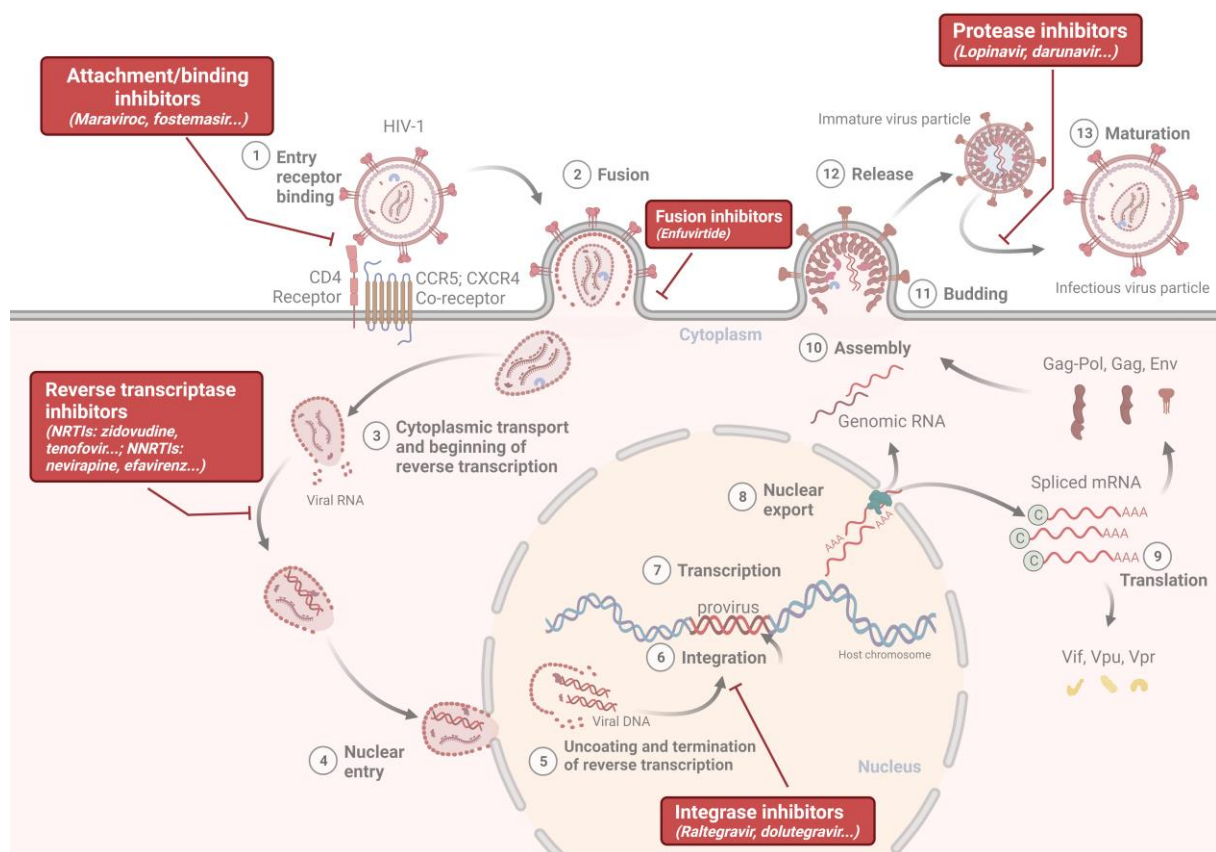
The initial wave of antibody response consists mostly of anti-gp41 IgG antibodies that form immunological complexes with HIV virions. However, although they bind infectious virus, anti-gp41 antibodies lack neutralizing activity<sup>52</sup>. By week three to four weeks of infection (Fiebig Stages III/IV), antibodies specific to additional HIV proteins, such as Gag, are developed. Anti-gp120 antibodies become the dominant antibodies after the transition from the acute to the chronic phase. Only 20% of PLWH produce broad neutralizing antibodies, which develop 2-4 years after infection. However, there is little evidence that these antibodies are effective in reducing viremia, since once HIV infection is established, the virus will eventually find ways to escape from the antibody responses<sup>53,54</sup>. Together, the aforementioned findings show limited effectiveness of antibody response during the acute phase in preventing HIV replication and transmission.

In HIV infection, HIV-specific T cell and antibody responses have been thoroughly studied. Despite these responses being orders of magnitude greater than those induced by other viruses establishing chronic infections, HIV-specific responses in the vast majority of PLWH fail to effectively control HIV replication and disease progression in the absence of ART. In HIV-exposed but uninfected people, immunoglobulin A (IgA) responses specific for HIV have been found in the mucosa<sup>55</sup>. These IgA might block HIV from infiltrating the epithelium. In natural control of HIV, protection can be linked to a number of genetic factors and the functional profile of the HIV-specific immune response, notably in the T cell response. When compared to progressors, CD8+ T cell responses from elite controllers (ECs) show a greater ability to produce several cytokines and chemokines, notably IL-2, to proliferate, to block HIV replication *in vitro* and kill target cells, to express perforin and degranulate in response to HIV-specific stimulation<sup>56</sup>. *In vitro*, viral suppression is mostly mediated by CD8+ T cells specific to Gag rather than Env or Nef, which is consistent with the observation that Gag-specific CD8+ T cell responses are associated with enhanced protection *ex vivo*<sup>57</sup>. Moreover, the existence of a reservoir of CD8+ T cells with immediate or fast inducible antiviral effector action is also a plausible difference, since CD8+ T cell responses from ECs reduce HIV replication without a prior *in vitro* activation<sup>58</sup>. There are also several studies that emphasize the importance of CD4+ T cell responses in HIV control. Enrichment in polyfunctional HIV-specific CD4+ T cells with an enhanced proliferative capacity, greater production of IL-21, higher functional avidity and T cell receptor (TCR) binding affinity, and the lower expression of CTLA-4, PD-1 and other exhaustion markers are some of the features associated with HIV control in ECs and long-term non progressors<sup>47,59-61</sup>. Overall, ECs CD4+ T cells do not exhibit immune fatigue symptoms including loss of polyfunctionality and expression of coinhibitory molecules<sup>62</sup>. As a result, these cells can release IL-2, IL-21, and other cytokines that aid CD8+ T cells in establishing and maintaining their effector capabilities, as well as their proliferation and survival capability<sup>63</sup>.

One of the many reasons why trials of HIV vaccines continue to fail despite extensive research is that we still do not understand what elicits an adapted and efficient immune response against HIV. This problem was not encountered in other infectious diseases that were the subject of vaccine development since natural control of these infections is not a rare event. Currently, there is no HIV vaccine available, and antiretroviral therapy is the sole effective treatment for HIV and AIDS.

## Antiretroviral therapy

The implementation of ART is still regarded as one of the most groundbreaking translations of fundamental molecular virology research to clinical biotherapy. Many antiretroviral drugs (ARVs) have been developed as a result of the tireless efforts of many experts in retrovirology, medicinal chemistry, enzymology, computational modeling, and structural biology to target various stages of the viral lifecycle; cell fusion and entry, and the activity of the three viral enzymes (PR, reverse transcription and IN) (**Figure 4**). These antiretroviral drugs are highly effective when used in combination therapy. Fostemsavir, a precursor of temsavir, is a medication that binds the HIV envelope glycoprotein gp120 and prevents virus attachment to CD4+ T cells<sup>64</sup>. Similarly, ibalizumab is a humanized monoclonal anti-CD4 antibody that can impede virus-receptor binding<sup>65</sup>. Maraviroc is a non-competitive, reversible CCR5 antagonist that also interferes with early steps of HIV infection<sup>66</sup>. Enfuvirtide belongs to the fusion inhibitors family of ARV. This molecule hinders the conformational changes affecting the HIV complex gp120/gp41 that facilitate fusion of the viral envelope and the host cell membrane<sup>67</sup>. Reverse transcription inhibitors account for more than half of all authorized medications. These molecules block the conversion of the viral single-stranded genomic RNA into double-stranded DNA, necessary for integration into host genomic DNA. Approved drugs targeting the RT's DNA polymerase activity include nucleoside analogues (i.e. nucleoside reverse transcription inhibitors (NRTIs)) and non-nucleoside reverse transcription inhibitors (NNRTIs).



**Figure 4. Major classes of antiretroviral medications and the reproductive Cycle of HIV-1.**

CCR5 co-receptor antagonists blocks viral binding to CCR5 and the fusion inhibitor, enfuvirtide, blocks fusion between the virus and the CD4 molecule. Reverse transcription the site of action of nucleoside and nucleotide reverse-transcriptase inhibitors (NRTIs) and non-nucleoside reverse-transcriptase inhibitors (NNRTIs). Integrase strand-transfer inhibitors (INSTIs) target this the integration step. Protease inhibitors interfere with viral proteins cleavage and inhibit the maturation of virions.

NRTIs are prodrugs that have to be converted to triphosphorylated derivatives before being incorporated into viral DNA. However, approved NRTIs lack a 3'OH in the ribose ring and act as chain terminators, blocking DNA synthesis. Within this class, tenofovir, an analogue of deoxyadenosine 5'-monophosphate monomer (dAMP), is often indicated as a first-line therapy in clinical guidelines<sup>68</sup>. NNRTIs are noncompetitive inhibitors that bind to an allosteric site located a few angstroms away from the RT's catalytic site<sup>69</sup>. The first medicine of this family to be licensed for clinical use was nevirapine, and efavirenz has been widely used for many years. In current formulations, second-generation inhibitors such as etravirine, rilpivirine, and doravirine have replaced first-generation NNRTIs. Two IN dimers assemble to two viral DNA ends and form a complex called the intasome. The intasome then binds to the cellular DNA and performs an integration reaction. First, the viral DNA ends are processed (3'-processing), and then a strand transfer reaction ligates the processed 3' ends of the viral DNA into the host chromosomal DNA. HIV integrase inhibitors (raltegravir, elvitegravir, dolutegravir, cabotegravir, and bictegravir) attach to the catalytic domain and prevent the strand transfer process from occurring<sup>70</sup>. Finally, HIV protease inhibitors are ARVs acting in the late phase of the replicative cycle of HIV. HIV protease cleaves viral precursor polyproteins to generate the mature protein components of an HIV virion. All approved protease inhibitors (saquinavir, lopinavir, darunavir, and others), except tipranavir, are peptidomimetics that act like a substrate analogs and compete with viral polyproteins. Instead of a peptide bond that would ordinarily be broken by hydrolysis, protease inhibitors contain a nonhydrolyzable hydroxyethylene core<sup>71</sup>. Combinations of two or three antiretroviral drugs were proven to durably suppress viremia to below the limit of detection of clinical tests, consistent with an arrest in viral replication<sup>72-74</sup>.

As discussed briefly above, although medical progress has been dramatic, there are still major gaps in care and need is ongoing. HIV is still a significant problem for worldwide public health as evidenced by the large number of new annual diagnoses. A global strategy plan in response to the ongoing HIV pandemic defined by UNAIDS is the "95-95-95" plan, which aims to identify >95% of HIV-seropositive people by 2025, treat >95% of them with ART, and achieve persistent virologic suppression in >95% of those treated. UNAIDS estimates that by 2030, the HIV pandemic will have decreased to a mild endemic condition if these goals are met by 2025<sup>75</sup>. However, due to the combined public health, economic, and political crises over the previous couple of years, UNAIDS stated earlier this year that the AIDS response is in jeopardy, with rising new infections and continuing deaths in many regions of the world<sup>76</sup>. Early on in the HIV epidemic, adherence to treatment was challenging due to a high pill burden that was also associated with several adverse effects<sup>77</sup>. With the introduction of ART, which combines three drugs into a single once-daily tablet regimen, allowing for reduced dosing and fewer adverse effects, high levels of adherence became easier to achieve. However, although largely improved, adherence to a daily oral drug intake remains a challenge in many cases. A potential strategy to address the adherence issue and prevent drug resistance, is the current development of long-acting formulations which could guarantee effective plasma concentrations for two months<sup>78</sup>.

Regular injections of long-lasting ART and implants are some of the suggestions that are thought to reduce the burden of ART and help make the treatment more sustainable for PLWH.

Nevertheless, the innovative delivery approaches and the improved pharmacokinetic profiles of the recent antiretrovirals do not improve the capacity of these molecules to eliminate persistently infected cells. Viral loads resume shortly after ART discontinuation, often within 2 weeks, even after decades of vigorous and continuous treatment<sup>79</sup>. Reactivation of the integrated proviruses is believed to be responsible for the rapid rebound viremia after cessation of ART. Prior studies estimated that the half-life of this pool of infected cells is 43.9 months,

or ~3.7 years<sup>80</sup>. At this pace, a population of one million infected cells would naturally decay to zero in 73.4 years, preventing people with HIV from being cured. This long-lived reservoir is often seen as the main barrier to finding a cure for HIV.

However, some exceptional cases of PLWH have been able to live with undetectable viremia despite interrupting ART, pointing to the possibility of HIV cure, or at least durable remission of HIV infection. The “Berlin Patient” (in 2009) and the “London Patient” (in 2019) were suffering from a hematological malignancy and both received allogeneic transplantation from donors with CCR5-deficient hematopoietic stem cells, a co-receptor used by HIV for viral entry<sup>19,20</sup>. The “Berlin patient” experienced HIV remission for 12 years and the “London patient” has been in HIV remission for ~3 years since the publication of this research. The proviral DNA was completely undetectable in these two persons making them the first two cases of what is considered “HIV cure” (complete eradication of the latent reservoir). While allogeneic stem cell transplant may have resulted in HIV eradication in these and other more recent cases (Düsseldorf, New York and City of Hope patients), it is not applicable on a large scale due to its complexity and the high risks. In addition, such interventions did not prove to be always successful<sup>81–83</sup>. Nevertheless, the successful cure of the “Berlin patient” and the “London patient” raised hopes for the possibility of curing HIV.

In other individuals, remission of HIV infection is related to their capacity to maintain undetectable viremia in the absence of ART. This status, also known as “functional cure”, is defined by the immune system’s ability to optimally combat infection in the absence of any interventions. ECs represent a small proportion of PLWH that can suppress plasma viremia to very low or undetectable levels for a prolonged period without ever receiving ART. Post-treatment controllers (PTCs) on the other hand are individuals who initially presented high viral loads and were put on ART for years before deciding to stop the treatment (for different reasons) and maintained since undetectable viremia. Many PTCs share the common feature of being treated early during primary infection (within 10 weeks after infection)<sup>84</sup>. While ECs and PTCs may share certain characteristics, there is mounting evidence that PTCs would not have been able to attain post-treatment control without the initial ART. The circulating reservoir in PTCs and ECs is generally substantially lower than in people on ART<sup>84–86</sup>. This small reservoir might be both a cause and a result of long-term immune control<sup>87</sup>. The HIV reservoir is at the heart of almost every cure strategy.

## **HIV cure strategies**

PTCs and ECs now serve as models for HIV remission and based on the observations made on these individuals, it is accepted that curative strategies should accomplish two main goals: i) reduction in the number of persistently infected cells and ii) induction of efficient immune control mechanisms to avoid viral spread from these cells. Thus, the search for therapeutic interventions that can eliminate or functionally control the HIV reservoir has high priority. In this next section, I describe a few of the methods currently being pursued to tackle HIV reservoir. These tactics can either be used to reduce the reservoir size, control viral rebound, or silence the reservoir.

## **Early antiretroviral therapy**

Early ART initiation within the first few weeks or months after infection is so far the only intervention that has been proven to significantly reduce the size of the reservoir. HIV reservoir seeding appears to happen very early in infection and early initiation of ART does not prevent the formation of a small pool of latently infected cells. Nonetheless, PTCs highlight the beneficial outcome of starting ART during the first weeks after infection. In addition, it is proposed that if people have unusually low reservoir sizes and present rapid decay rates<sup>88,89</sup>, it remains possible that some will be cured after decades of ART. With the development of new antivirals, it is possible that potent compounds with the capacity to penetrate all tissue reservoirs will accelerate the decay for the virus, leading eventually to a cure in some individuals. However, given the slow rate of virus decay during ART, current therapies are unlikely to be curative even when initiated very early. Other advantages of early ART initiation are the limitation of viral diversity and the preservation of immune responses. However, the probability to maintain undetectable viremia after interruption of early initiated ART is estimated at ~6%, and additional strategies will be needed to achieve durable HIV remission in a large proportion of PLWH.

## **Latency Reversal (shock and kill)**

“Shock and kill” approach uses latency reversal agents to increase HIV transcription/replication and virions production. This strategy target the molecular mechanisms maintaining latency with the goal of efficiently reactivating latent proviruses (“shock”) and eliminating the productively infected cells (“kill”). It is hypothesized that upon viral reactivation, infected cells will be killed either as a result of the virus' cytopathic effects or after recognition and elimination by immune cells<sup>90,91</sup>. The bulk of latency reversal agents have extremely variable activities depending on the evaluated *in vitro* latency model, HIV integration site, CD4+ T cell subset and often fail to stimulate the formation of viral particles in cells of PLWH, and are unable to significantly reduce the size of the HIV reservoir *in vivo*. Latency reversal agents are often extremely toxic and can only be used for a very short period of time<sup>92</sup>. Romidepsin is a histone deacetylase inhibitor (HDAC inhibitor) that has been authorized for the treatment of cutaneous and peripheral T cell lymphoma. It is one of the most effective and thoroughly clinically studied latency-reversing medicines available<sup>93,94</sup>. However, in a recently reported dose-escalation trial, the use of this HDAC inhibitor did not result in noticeable latency reversal in individuals on ART<sup>95</sup>. Despite numerous attempts being made to discover new classes of latency reversal agents and techniques aimed at eliminating cells harboring the reactivated provirus, the elimination of reactivated cells is very inefficient in people on suppressive ART. This could be caused by low amount of expressed viral antigens, enhanced resistance to cell death of persistently infected cells or scarce numbers of HIV-specific cytotoxic immune cells.

## **Latency Silencing (block and lock)**

“Block and Lock” strategy is fundamentally different from the “Shock and kill” approach. Using latency promoting agents to “block” viral transcription and “lock” HIV promotor in a latent state through repressive epigenetic alterations, this technique seeks to permanently mute

the reservoir. Latency promoting agents act by inhibiting viral or cellular proteins involved in HIV transcription<sup>96</sup>. Deep latency of an integrated retrovirus would not be unprecedented. A sizeable portion (~8%) of the human genome is composed of ancient retroviruses that are seldom, if ever, translated. Human endogenous retroviruses are silenced through epigenetic mechanisms. This supports the “Block and Lock” strategy feasibility and possible sustainability. Still, the efficacy of latency promoting agents at preventing viral rebound in PLWH, in the absence of any pharmacological intervention, has not been investigated yet. Further research is needed to advance its potential or understand limitation.

## **Immunotherapy**

This family of approaches targets the reservoir indirectly by eliciting sustained immune responses specific to HIV that are capable of efficiently eliminating infected cells during ART and/or inhibiting reservoir reseeded upon ART interruption. Such approaches include broadly neutralizing antibodies (bNAbs) and HIV-specific chimeric antigen receptor (CAR)-T cells<sup>97,98</sup>. Both bNAbs and CAR-T cells have potential limitations in that they both require the epitope to be presented at high densities on the cell surface and both target the extremely variable envelope glycoprotein. Thus, these approaches may only work when used in high viral replication settings, such as in combination with latency reversing agents or during a treatment cessation. Furthermore, the passive administration of potent bNAbs has also been found to select for variants with mutations in multiple antigenic sites, which leads to a loss of HIV control, even if it initially provides favorable clinical outcomes in humanized mice and humans<sup>99–102</sup>. Therefore, it is probable that monoclonal antibodies alone would not effectively eliminate the infection. Leveraging advances made in the management of cancer, several groups are studying immune checkpoint blockade. In addition to their role in HIV-associated immune exhaustion, immune checkpoints also identify cells that are enriched for HIV<sup>103</sup>. Antibodies directed against immune checkpoints such as PD-1 and CTLA-4 promote latency reversal and boost antigen-specific T cell responses<sup>104,105</sup>. In fact, immune checkpoint inhibitors are an example of compounds that have been implicated in the transcriptional process of HIV and latency disruption, hence their use in “shock and kill” therapeutic strategies. There are studies showing that the administration of anti-PD-1 to PLWH on ART resulted in a significant increase in HIV RNA, and consequently, reversal of HIV latency<sup>105</sup>. Moreover, several immune checkpoint molecules, such as PD-1 are found to be upregulated in patients who are not on ART<sup>105</sup>. Although immune checkpoint blockade led to a marked decrease in markers of HIV persistence in case report studies, two clinical trials using anti-PD-1 and anti-PD-L1 (programmed cell death-ligand 1) were prematurely terminated due to immune-related adverse events<sup>106–108</sup>.

## **Gene Editing**

The excision or inactivation of the HIV genome is now potentially achievable thanks to the recent developments of the CRISPR/Cas9 technology<sup>109</sup>. Several *in vitro* models were used to evaluate the inactivation of the provirus by targeting several conserved HIV sites. Although the strategy seemed promising at first, it led to a long-term escape driven by HIV DNA mutations in the targeted regions<sup>110,111</sup>. Alternatively, disruption of the virus co-receptor CCR5 and other

pathways required for virus replication may contribute to render cells less susceptible\* to infection; in these circumstances, gene modifications may not need to be 100% successful, as merely limiting the number of vulnerable targets may be sufficient to significantly reduce the spread of the virus<sup>112</sup>.

There remains an ongoing global public health priority to find an efficient and scalable cure for HIV. Despite advances in both prevention and therapy, there is still a great need for a long-term treatment. Technological improvements have made great strides toward understanding the biological properties of the HIV reservoir since the initial description in 1997. However, HIV cure initiatives are impeded by inadequate methods for assessing the viral reservoir in people receiving ART and limited characterization of the cells supporting HIV replication *in vivo*. Understanding the cell types and anatomical sites that persistently harbor HIV proviruses is essential for the design of therapeutic strategies to eradicate HIV from PLWH.

## Target cells and HIV reservoir

The HIV reservoir is defined as a cell type or anatomical site in which a replication-competent virus accumulates and persists, throughout lengthy periods of ART-suppressed viremia<sup>113</sup>. The reservoir is constituted by long-lived cells that harbor integrated and replication-competent provirus in individuals on ART. These cells cannot be recognized and eliminated by the immune system and constitute a major obstacle to virus elimination. In addition, it has been established that even under suppressive ART, active virus replication continues in select sites<sup>113</sup>. These reservoirs also strongly contribute to the problem of viral persistence on treatment.

### Anatomical reservoirs

Since it infects immune cells, HIV dissemination in the lymph nodes (within days) and bloodstream (within weeks) is inevitable<sup>114</sup>. HIV spreads by the transport of infected immune cells, by cell-to-cell transmission and by progeny virus production<sup>115</sup>. Peripheral blood contains less than 2% of the body's total T cells and circulating cells do not serve as the exclusive site of HIV replication. Hence, the discovery of HIV-infected cells in several organs following years of ART is not unexpected<sup>116,117</sup>. HIV-infected cells have been detected at high frequency in the gastrointestinal tract and lymphoid tissues, as well as at lower frequencies in the brain and central nervous system, the lungs, kidneys, liver, spleen, adipose tissue, the male and female genitourinary systems, and bone marrow<sup>118</sup>.

Several groups are now mapping the spatial distribution of HIV throughout the body. The largest amount of virus and highest frequency of infected cells are found in tissues that are significantly enriched with lymphoid structures, notably lymph nodes and the mucosa of the digestive tract<sup>119-121</sup>. In a thorough analysis of the reservoir in SIV-infected macaques receiving successful ART, the gut was shown to contain the vast majority (>98%) of the whole body reservoir<sup>122</sup>. It is also assumed that the largest HIV reservoir in human is the gut<sup>120,122</sup>.

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\* The terms susceptible and permissive are used interchangeably in this manuscript.

Within these compartments, HIV is found in T lymphocytes and macrophages but also its presence has been suggested in organ-specific cell types and HIV has been reported to infect a wide range of cells *in vitro*<sup>118,123,124</sup>. However, because the only cell types *in vivo* that are consistently found to be infected with HIV are CD4<sup>+</sup> T lymphocytes and cells of the monocytes/macrophage lineage, the relevance of *in vitro* viral replication in other cell types to HIV disease is unclear at present and still needs to be further investigated.

## **Cellular reservoirs**

The establishment and maintenance of HIV reservoirs is a complex process that is determined by three key factors: (1) the cell's susceptibility and permissibility to HIV infection, (2) the infected cell's ability to survive cytopathogenic effects of the infection and escape immune surveillance, and (3) the infected cell's life span and turnover potential.

HIV, as well as the related primate lentiviruses HIV-2 and SIV, infect cells that express CD4 and an adequate chemokine receptor, primarily CCR5 and occasionally CXCR4 or other receptors. Myeloid cells also express HIV primary receptor CD4 and the chemokine co-receptor CCR5, and are also infected *in vivo* by R5-tropic and dual tropic strains of HIV. However, the magnitude of HIV infection depends on the type of cell. Human peripheral CD4<sup>+</sup> T cells are more vulnerable to HIV infection than macrophages or DCs. It is generally accepted that memory CD4<sup>+</sup> T cells represent the major cellular reservoir for HIV, at least quantitatively<sup>125,126</sup>. To a lesser extent, cellular reservoirs may also comprise myeloid cells such as monocytes, macrophages, and DCs<sup>124,127</sup>.

### **DCs**

DCs are a heterogeneous population of antigen-presenting cells (APCs) that can be lymphoid or myeloid in origin and have different properties depending on their anatomical location. They serve as a link between the innate and adaptive immune systems. They develop and migrate to lymph nodes after antigen recognition to present antigen to T lymphocytes. Myeloid DCs, pDCs, and Langerhans cells are the three main subtypes of DCs, and all three subtypes are susceptible to HIV infection *in vitro* but appear to be less so than CD4<sup>+</sup> T cells *in vivo*. However, there are studies showing that DCs, and particularly follicular DCs, may also be important components of HIV persistence<sup>128–130</sup>. Follicular DCs in B follicles are derived from perivascular progenitors and can contribute to HIV persistence by sequestering a stable pool of HIV virions on their surface without necessarily being infected, and putting them in contact with CD4<sup>+</sup> T cells<sup>131</sup>, hence facilitating *trans* HIV infection in these cells<sup>130</sup>. Moreover, Haase *et al.* showed that in ART-treated PLWH follicular DCs presented on their surface a relatively stable pool of virions<sup>132</sup>. This was supported by further research, which indicated that follicular DCs are able to induce low-level viremia and recruit resting CD4<sup>+</sup> T cells, which helps in virus spread. Because the trapped virions are replication-competent, follicular DCs may therefore play a role in HIV pathogenesis as well as act as a driver in HIV reservoir establishment and persistence in resting memory cells<sup>133–135</sup>.

### **Macrophages**

Tissue-resident macrophages are located in different anatomical areas and organs. In response to inflammatory signals, monocytes can migrate to tissues and acquire the phenotype of tissue-resident macrophages. However, embryonically derived macrophages (Kupffer cells,



microglia, Langerhans cells, lung alveolar macrophages, and splenic red pulp macrophages) that seed tissues as progenitor cells throughout embryonic development are long-lived and capable of self-renewal independently of monocytes. *In vitro*, depending on the types of the stimuli and the culture medium that macrophages are exposed to, these cells can be polarized into distinct phenotypes. Initially, these polarized states were divided into the classically activated M1 macrophages and the alternatively activated M2 macrophages. M1 macrophages exhibit inflammatory roles while M2 macrophages possess anti-inflammatory functions. It is recognized however, that macrophages exhibit high plasticity and vary considerably according to which stimuli they are subjected to, which hampers the attempts of their classification *in vivo*<sup>136</sup>.

There are three predominant subtypes of circulating monocytes: classical, intermediate and non-classical monocytes. Classical monocytes, expressing low levels of CD4 and CCR5 receptors, are less susceptible to HIV infection than intermediate or non-classical monocytes. Macrophages also express both the entry receptor CD4 and co-receptors CCR5 and CXCR4 and were demonstrated capable of supporting viral replication in PLWH<sup>137,138</sup>. HIV DNA has been detected in monocytes after acute infection, and they may be crucial in the spread of the virus to the brain and other tissues since they migrate from the blood to seed many different organs<sup>139</sup>. The contribution of these infected monocytes in the spread and seeding of HIV reservoir is unknown as their half-life may be shorter than embryonically derived macrophages. On the other hand, perivascular macrophages have a longer life span and they can also become infected. Perivascular macrophages are highly mobile compared to other tissue-resident macrophages and can infiltrate other organs.

Most of macrophages are found in hard-to-access sites and their role as a reservoir during ART remains to be clearly elucidated<sup>124,140,141</sup>. Nonetheless, replication competent virus was recovered from cultures of macrophages derived from lymphoid tissues of acutely treated macaques<sup>142-144</sup>. Similarly, urethral macrophages in ART-suppressed PLWH were also shown to harbor HIV reservoir in penile urethra<sup>145</sup>. In these macrophages, containing total and integrated HIV DNA, lipopolysaccharide (LPS) stimulation reversed latency leading to expression of HIV p24 protein<sup>145</sup>. There is evidence that macrophages can remain infected for long periods as they have an extended life span and are less susceptible to the cytopathic effects caused by HIV compared to CD4+ T cells<sup>128</sup>. A growing body of evidence indicates that macrophages are HIV particles-storing cells with lengthy viral half-lives. Additionally, unintegrated viral DNA can be found in metabolically active macrophages, where it can remain stable and active for up to two months in non-dividing macrophages<sup>146</sup>. Unintegrated HIV DNA in macrophages may therefore contribute to HIV reservoir. Importantly, macrophages, like DCs, are APCs, and viral replication in these phagocytic cells may increase HIV transmission to CD4+ T cells not only via the virological synapse, but also via the production of chemoattractant cytokines, which facilitates the recruitment and activation of other immune cells, CD4+ T lymphocytes included<sup>147,148</sup>. Macrophages also represent a source of virus that is inaccessible to the immune system<sup>149</sup>. Overall, the presence of HIV in macrophages was demonstrated many years ago<sup>150</sup>, although their origin is a highly debated topic<sup>151,152</sup>, their role in HIV pathogenesis and their contribution to the reservoir received more attention in the recent years. Whether they directly become infected or they engulf infected CD4+ T cells<sup>153</sup>, it is reasonable to suspect that macrophages contribute to the dynamics of HIV infection *in vivo*, as

the virus evolve to become macrophage-tropic with disease progression<sup>154</sup>. Due to their innate cellular and immunological properties, macrophages and DCs may be significant contributors to viral maintenance and rebound even if their contribution to the latent reservoir appears to be less than that of CD4+ T cells<sup>128,155</sup>.

### CD4+ T cells

CD4+ T cells remain the best-characterized reservoirs for HIV in virally suppressed PLWH on ART. CD4-CD8- T cell precursors (thymocytes) emerge from the bone marrow and migrate to the thymus where they undergo TCR gene rearrangement and antigenic selection, two critical steps in the development of mature T-lymphocytes. Thymocytes express surface markers that are called clusters of differentiation (CD). CD3, CD4 and CD8 are characteristic of T lymphocytes. By the end of the maturation process, thymocytes become mainly CD3+CD4+CD8- (CD4+ T cells) or CD3+CD4-CD8+ (CD8+ T cells). Mature naïve CD4+ T cells exit the thymus and are then deployed to secondary lymphoid organs, including the spleen, lymph nodes, and the mucosa-associated lymphoid tissue, where they constantly survey for peptide-MHC II (pMHC) molecules, for antigen recognition. The survival of these cells is notably dependent on signaling induced by self-peptide/MHC complexes and TCR interaction, and interleukin-7 (IL-7). The viral genome is not efficiently reverse transcribed in quiescent cells, in contrast with activated CD4+ T cells, indicating that at least a minimal level of activation is necessary to establish infection. CD4+ T cells with low levels of activation are mostly found in tissues, even in homeostatic conditions. CD4+ T cells do not represent a homogeneous pool of target cells. Instead, individual subsets of CD4+ T cells are thought to be differently infected by the virus *in vivo*.

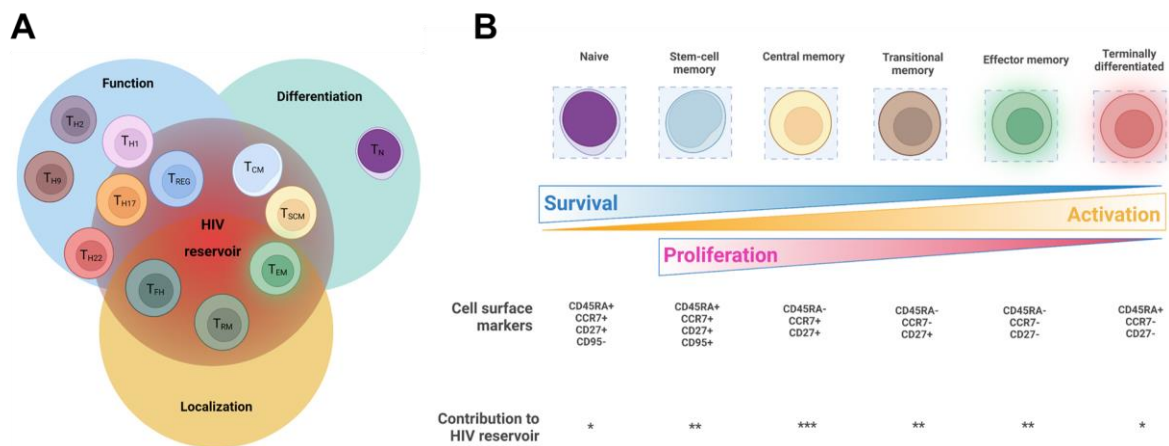
### Function and differentiation

Differentiation and activation of T cells is dependent on three signals: (1) the engagement of the antigen receptor by a pMHC complex, (2) co-stimulation, and (3) cytokine stimulation. TCR coupled with CD3 activation consequently induces a network of downstream signaling pathways, which drive naïve T cells to proliferate and differentiate into effector or memory T cells.

The effector function of CD4+ T cells is elicited by the expression of specific transcription factors, chemokine receptors, and cytokine production in response to stimulation, which all influence the cell's capacity to serve as a long-term reservoir for HIV. Along these lines, certain functionally polarized subsets of CD4+ T cells are found to be specifically enriched in HIV persistent infection (**Figure 5A**). Upon activation through TCR, naïve CD4+ T cells differentiate into lineage specific T helper (T<sub>H</sub>) subsets. Each subset produces distinct sets of cytokines that activate downstream signal transducer and activator of transcription (STAT) signaling proteins and dictate lineage commitment by expressing unique master transcription factors<sup>156</sup>. TH cells are typically classified by the cytokines they produce upon TCR engagement. T<sub>H1</sub>, T<sub>H2</sub>, T<sub>H9</sub>, T<sub>H17</sub>, T<sub>H22</sub>, T<sub>REG</sub>, and T<sub>FH</sub> cells are all functional subsets of the CD4 compartment that are defined various functional characteristics and homing capacities. T<sub>H17</sub> cells are highly sensitive to HIV infection<sup>157</sup>. Although, several studies suggested that tissue-based T<sub>H17</sub> cells are enriched for HIV DNA, mostly because they are found in the gut, which is a hotspot for virus replication<sup>158</sup>, recent work from Renault *et al.* showed that T<sub>H17</sub> are enriched for different intrinsic factors that are essential for viral replication<sup>159</sup>. Moreover, blood-derived T<sub>H17</sub> are also enriched in HIV provirus<sup>160</sup>. Additionally, T<sub>H17</sub> cells have stemness characteristics that contribute to their long lifespan. Such features enable T<sub>H17</sub> cells' potential to act as a long-lived viral reservoir for HIV<sup>161</sup>. The B cell follicle within lymphoid structures has unique

properties that make it ideal to support HIV replication and persistence. Within the B cell follicles, in states of inflammation,  $T_{FH}$  can become activated and enriched in replication-competent proviruses<sup>119</sup>. Further studies have also identified  $T_{H1}$  as potential reservoir populations with large portion of replication-competent viral genomes<sup>162,163</sup>. Different investigations showed that  $T_{REG}$  cells are also highly enriched in HIV DNA and can generate infectious virus<sup>164,165</sup>.

Activated  $CD4^+$  T cells are the preferential target for productive HIV infection. These cells are not found to be the major concern in HIV persistence research, due to the short lifespan. Effector  $CD4^+$  T cells provide important functions designed to clear inciting antigens but typically exhibit a short life span delimited by activation-induced cell death. However, some activated effector cells have the ability to revert to a resting state and differentiate into long-lived memory cells<sup>126</sup>. After antigen clearance, a small percentage of these cells remain as memory cells, which are maintained for decades in response to homeostatic signals like IL-7. Memory  $CD4^+$  T cells are characterized by the ability to serve as permanent supply for subsequent antigen-induced activation into effector cells and a high proliferative potential on subsequent exposure to antigen and a long-life span. The memory compartment is heterogeneous and has been conventionally categorized into T stem cell memory ( $T_{SCM}$ ), central memory ( $T_{CM}$ ), transitional memory ( $T_{TM}$ ), migratory memory ( $T_{MM}$ ), tissue resident memory ( $T_{RM}$ ), effector memory ( $T_{EM}$ ), and terminally differentiated ( $T_{TD}$ ) cells<sup>166</sup>.



**Figure 5. Contributions of  $CD4^+$  T cells subsets to HIV persistence and schematic representation of the characteristics of different  $CD4^+$  T cell subset.**

(A)  $CD4^+$  T cell subsets can be classified according to their functions (blue), memory status (green) and localization (yellow). Some of these classifications largely overlap. The relative contributions to viral persistence is depicted by their proximity to the red zone representing the HIV reservoir (adapted from From Fromentin R and Chomont N. *Semin Immun*, 2021<sup>913</sup>). (B) The relative lifespan of each subset is represented in blue, with naive T cells presenting better survival capacities, and the terminally differentiated T cells representing the shortest lifespan. Similarly, the activation status and proliferative capacities are represented in yellow and red respectively. The phenotype based on surface cell marker expression of each subset are also shown, as well as relative contribution of each cell subset to the HIV reservoir (\*: lowest contribution; \*\*\*: highest contribution).

Studies have shown that the distinct CD4 memory T cell subsets may support viral persistence through various mechanisms<sup>126</sup>. Memory cells that harbor intact replication-competent provirus are rare but extremely stable<sup>167</sup>. Although there are no cell surface markers that unambiguously distinguish all different T cell subsets, in general, CD45RA, CCR7, CD27 and CD95 molecules are commonly used to identify the different T cell subsets (**Figure 5B**)<sup>168</sup>.

Naïve T ( $T_N$ ) cells have not encountered yet their cognate antigen.  $T_N$  cells are characterized by the expression of CD45RA and do not express CD45RO which is a memory-associated marker<sup>169</sup>. They also express lymph node homing receptors CCR7, CD27, and are negative for CD95. Naïve T cells are actively maintained in a state of hypo-responsiveness, which is characterized by a small cell size, a low proliferative rate, and low basal metabolism. Upon activation,  $T_N$  cells downregulate CD45RA expression and differentiate into effector cells, which readily release cytotoxic granules and effector cytokines upon engagement of their TCR with cognate antigen. Although naïve cells can harbor HIV DNA and replication-competent HIV, their infection rate is generally significantly lower than that of memory CD4+ T cells<sup>126,170</sup>. However, naïve cells can potentially contribute to HIV persistence as a result of their longevity<sup>171,172</sup>, even though memory cells typically contain the majority of the HIV proviral DNA throughout ART<sup>173</sup>.

$T_{SCM}$  are considered the least differentiated stage of memory T cell. This subset of antigen-experienced cells comprise a relatively rare memory population (2-4% of circulating lymphocytes) having a largely  $T_N$  phenotype, being CD45RA+CCR7+CD27+, while overexpressing the CD95 antigen, which is expressed at high levels by memory cells. This subset is long-lived and has the ability to differentiate into  $T_{CM}$ .  $T_{SCM}$  are permissive to HIV infection and the proportion of  $T_{SCM}$  cells containing HIV DNA is higher than in  $T_N$ ,  $T_{CM}$  and  $T_{EM}$ <sup>174</sup>. Despite being a relatively rare population,  $T_{SCM}$  are extraordinarily stable over time and are kept alive by homeostatic self-renewal, making them a sustainable HIV reservoir.

$T_{CM}$  are phenotypically defined as CD45RA-CCR7+CD27+. They are also long-lived and show exquisite survival and self-renewal abilities.  $T_{CM}$  are characterized by their extremely low degree of cellular proliferation in basal conditions, which makes their recognition and elimination by the immune system unsuccessful. Furthermore, pro-survival pathways are especially activated in  $T_{CM}$  cells<sup>175</sup>. Because of these characteristics,  $T_{CM}$ , along with  $T_{SCM}$ , are believed to be the most obstinate source of virus during long-term ART.  $T_{CM}$  are still regarded as the most important contributor to the persistent HIV reservoir in the majority of PLWH.

$T_{TM}$  cells are more differentiated than  $T_{CM}$  cells but not as fully differentiated as  $T_{EM}$  cells in terms of phenotype.  $T_{TM}$  are CD45RA-CCR7-CD27+.  $T_{TM}$  are also a major reservoir for HIV DNA and can expand by homeostatic proliferation<sup>126</sup>. HIV provirus in  $T_{TM}$  was shown to be less frequently replication-competent than in  $T_{CM}$ , indicating that there is an excess of defective viruses that are possibly expanded through proliferation of precursor cells<sup>176</sup>.

$T_{EM}$  are characterized by a more differentiated phenotype, higher rates of proliferation, and a shorter life span than its precursor cells. They are phenotypically defined as CD45RA-CCR7-CD27-. The  $T_{EM}$  subset may encompass the majority of intact and potentially replication-competent proviruses<sup>177</sup>.

$T_{CM}$ ,  $T_{TM}$ , and  $T_{EM}$  cells are considered the three main HIV reservoirs in PLWH receiving suppressive ART (**Figure 5B**)<sup>126</sup>. However, their relative contribution to the functional reservoir varies<sup>176</sup>. Infected memory CD4+ T cells from different memory subsets exhibit varying levels of HIV transcriptional activity, proviral inducibility, and contribution to the pool of cells with genetically intact genomes<sup>158</sup>.

### Phenotype

An important topic of current study is the accurate characterization of cells harboring proviruses. If a characteristic phenotype can be defined and validated, treatments to eradicate the specific reservoir cells could be developed. This has been challenging due to the scarcity of infected cells, but substantial progress has been achieved by several separate groups. Given the significance of cell activation and proliferation in reservoir maintenance, HIV proviruses are abundant in cells that exhibit common markers of activation, such as HLA-DR (major histocompatibility complex [MHC] class II)<sup>178,179</sup>, CD25 (the  $\alpha$  chain of the IL-2 receptor and a constitutive hallmark of regulatory T cells)<sup>165</sup>, and CD69 (a marker of tissue-resident memory T cells)<sup>180</sup>. Therefore, HLA-DR, CD25, or CD69-expressing cell subsets that do not fit the traditional description of resting CD4<sup>+</sup> T cells are involved in the long-term persistence of HIV during ART. As mentioned in the section above, HIV proviral DNA has been observed to be moderately enriched in cells expressing immunological checkpoint receptors, such as PD-1, CTLA-4, T cell immunoglobulin and ITIM domain (TIGIT), lymphocyte activation gene 3 (LAG-3), T cell immunoglobulin and mucin 3 (TIM-3), and CD160<sup>158,181,182</sup>. Importantly, the more receptors were expressed, the greater the level of enrichment was<sup>181,182</sup>. CD32a has also been proposed as an HIV reservoir marker<sup>183</sup>, but this finding has been challenged in subsequent reports and still remains controversial<sup>184</sup>.

Subsets defined based on effector function, differentiation status as well as the expression of specific markers, all reflect the intrinsic characteristics of a cell. During the maturation or polarization process, cells change their intracellular rewiring to adapt to their new functions. Those adaptations necessitate the upregulation or downregulation of certain factors that may accidentally help promote or block HIV infection. For instance T<sub>H17</sub>, which are preferential cellular targets for HIV, are enriched for different factors that essential for virus replication<sup>185</sup>. Also, the productive infection of both naïve and memory CD4<sup>+</sup> T cells with HIV, requires a progression to the G1b stage of the cell cycle<sup>186</sup>. Most circulating peripheral T lymphocytes and many in lymphoid tissues are in a G0 resting state, the quiescent phase of the interphase (composed of G0, G1, S, and G2). The first stimulation signal, delivered by the antigen-specific TCR, causes an increase in the expression of certain lymphocyte activation molecules, the activation of lymphokine genes and cellular oncogenes required for cell division, as well as a cell cycle transition from the G0 to the G1 stage. Progression from G0 to G1b elevates certain blocks to HIV replication and allows the expression of certain factors necessary for the viral cycle<sup>187</sup>. Such factors are known respectively as restriction factors and HIV dependency factors (HDFs).

## **Molecular determinants of HIV permissibility and persistence**

The genome of HIV encodes only 19 proteins, consequently, it has to rely on the host machinery and proteins to complete its replication cycle. It has long been known that viruses are strictly dependent on live host cells in order to replicate. However, it has become clear that these interactions are complex and depend on a delicate balance between host proteins and complexes that are necessary for or facilitate viral replication (HDFs) (**Figure 6**), and components of intrinsic immunity of a cell, termed restriction factors (RFs), which constitute the first line of defense by blocking different steps of the viral replication cycle (**Figure 7**).

## Host-dependency factors necessary for HIV life cycle

HDFs are the explanation behind why HIV preferentially replicates in activated CD4<sup>+</sup> T cells and less efficiently in macrophages and immature DCs, and what makes resting CD4<sup>+</sup> T cells, monocytes, and mature DCs relatively resistant to HIV infection. Indeed, infection of quiescent T cells is blocked before integration, probably due to incomplete reverse transcription or failure to transport the viral PIC to the nucleus<sup>188,189</sup>. It became evident through categorization of HDFs that HIV substantially depends on several biological processes, including cell activation, cell cycle progression, proliferation, and metabolism. Early studies suggested that cell activation, as defined by cell cycle progression beyond G1b, is not only required for proviral production but also for efficient reverse transcription and proviral integration<sup>186</sup>. Progression from G0 to G1b cell cycle provides the cells with cellular cofactors, cellular pathways as well as chromatin access needed for the viral cycle. The discovery that CCR5 gene defect has a major impact on susceptibility to HIV infection and on disease progression in HIV prompted the observation that certain host factors are crucial to viral replication and their absence could be deleterious to the infection. To date, there have been hundreds of identified HDFs<sup>190,191</sup>. In the next section, we will discuss briefly few major factors that were best characterized and confirmed by different studies (**Figure 6**).

### Viral entry

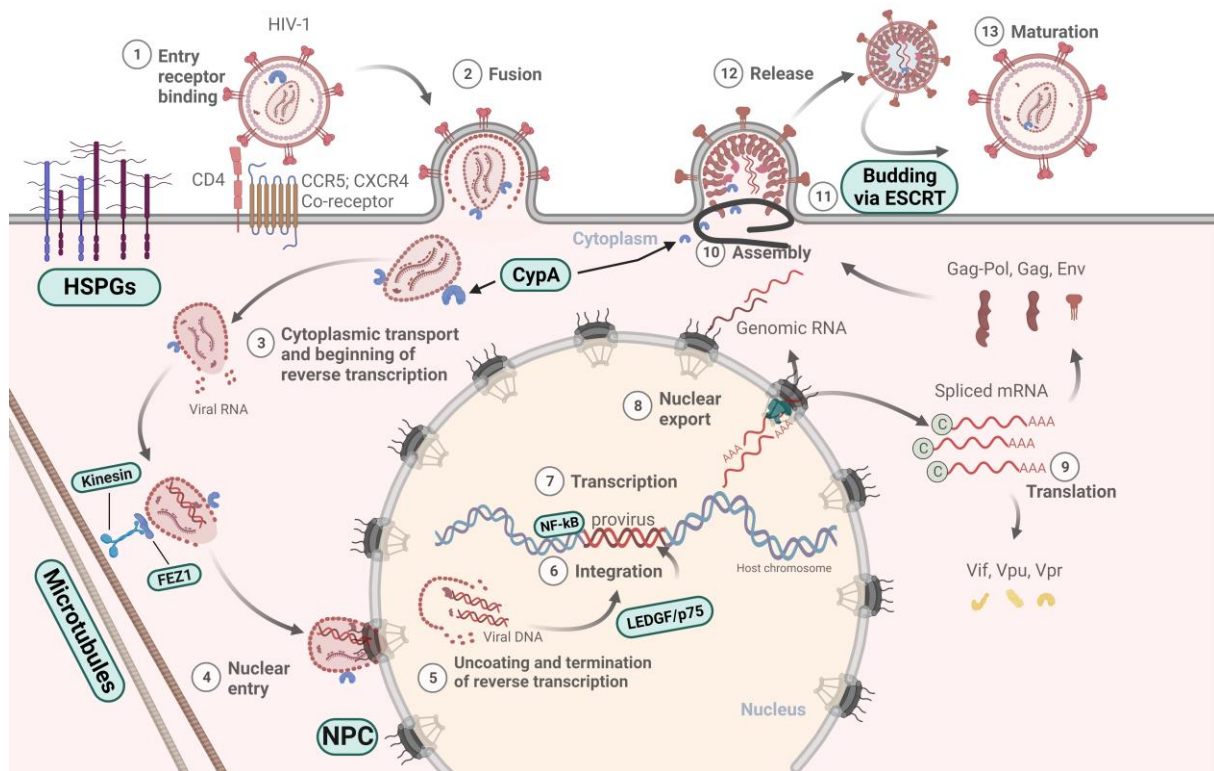
HIV requires attachment to non-receptor, cell surface molecules, which provides an important opportunity to increase encounters with entry receptors. The first biologically relevant non-receptor interaction discovered was binding to heparan sulfate proteoglycans (HSPGs) on macrophages (**Figure 6**)<sup>192</sup>. DCs were also shown to capture HIV via scavenger receptors such as DC-SIGN, Siglec 1, Syndecan 3 and others<sup>193-195</sup>.  $\alpha 4\beta 7$  integrin on peripheral T cells has also been implicated in HIV entry<sup>196</sup>. For cell entry, HIV requires binding to its primary receptor, the host protein CD4 followed by binding to a co-receptor such as CCR5 (for R5-tropic HIV) or CXCR4 (for X4-tropic HIV) that would trigger membrane fusion between the viral and the cell's membrane. Although the expression of CD4 is ubiquitous among all CD4<sup>+</sup> T cell subsets, the expression of chemokine co-receptors CCR5 and CXCR4 is modulated by the differentiation process, which contributes to the cells' susceptibility to HIV.

### Reverse transcription

After the fusion step, cyclophilin A (CypA) interacts with the viral capsid in the cytoplasm. CypA was shown to be incorporated into viral particles via its interaction with Gag, and enhance viral infectivity by helping to disassemble the capsid core structure immediately following the infection of a new cell<sup>197</sup>. Other studies showed that CypA promotes capsid stability by delaying uncoating and promoting reverse transcription and integration<sup>198</sup>. CypA binding to HIV CA promotes an early step in the HIV replication cycle in specific target cells. In addition, high levels of dNTPs in activated cells allows the virus to establish infection more efficiently. For example, high levels of dNTPs in activated CD4<sup>+</sup> T cells (2-5  $\mu$ M) allows for efficient HIV reverse transcription upon entry<sup>199</sup>. On the other hand, dNTP levels are low in resting CD4<sup>+</sup> T cells and myeloid cells, which reduces but not blocks efficient viral reverse transcription and replication<sup>199,200</sup>.

### Trafficking and integration steps

Signals from the gp120 receptor interaction, in particular with the chemokine co-receptors, causes cytoskeleton rearrangements, which are necessary for intracellular trafficking of the virus for both nuclear import and nuclear export<sup>201</sup>. Interaction between HIV and microtubule-based motors is facilitated by CA-binding adaptor proteins. For several decades, retroviral core uncoating has been thought to occur in the cytoplasm in coordination with reverse transcription<sup>202</sup>. However, it was shown that HIV trafficked to the cell nucleus via microtubules and that this step was dependent on CA<sup>203</sup>. Bicaudal D2 (BICD2) is a dynein motor adaptor protein that associates directly with HIV CA<sup>204</sup>. Fasciculation and Elongation protein Zeta 1 (FEZ-1) is a kinesin-1 adaptor protein that binds to HIV capsid-nucleocapsid assemblies and is required for HIV transport towards the periphery of the cytoplasm<sup>205</sup>. Loss of BICD2 results in almost complete abrogation of microtubule-based transport of the CA to the nucleus in infected cells *in vitro* and knockdown of FEZ1 results in loss of HIV nuclear accumulation, demonstrating the importance of these two cellular proteins for HIV infectivity<sup>204,205</sup>. This was early evidence suggesting that some CA was present up until the viral complex reached the nucleus. Interaction between the CA and intracellular trafficking proteins was also shown to be important in shielding the virus from detection by the innate immune system<sup>206</sup>.



**Figure 6. The capsid protein interacts with several host dependency factors.**

Host proteins that have a role in the replication cycle and are discussed in the manuscript are indicated. The host cellular protein CypA binds to capsid and maintain core stability during cytoplasmic trafficking. The capsid binds the microtubules motor adaptor FEZ1 to bridge viral particles kinesin-1 motors, which mediate their long-range bidirectional transport on stable microtubules towards the nucleus. When capsid arrives at the nuclear envelope, it binds NUPs and other factors, to promote import into the nucleus via the nuclear pore complex as an intact or nearly intact capsid. Reverse transcription is completed in the nucleus, followed by uncoating of the capsid. PIC-associated integrase orchestrates the formation of the integrated provirus, aided by LEDGF. Proviral transcription mediated by host RNA polymerase II (RNA Pol II) and P-TEFb, yields viral mRNAs. Viral-particle budding and release from the cell is mediated by ESCRT complexes and ALIX.

Following cytoplasmic trafficking, the CA localizes to the nuclear pore complex (NPC), allowing the HIV viral DNA to enter the nucleus. Of note, the NPC provides the sole conduit for viral particle translocation into the nucleus during cell interphase (as discussed above). It was later discovered that in addition to its hijacking of the cellular microtubule network, the CA would also interact with nucleoporins (NUPs) contained in the NPC and that this interaction was vital for nuclear import of the viral PIC. Many NUPs, including NUP358, NUP214, NUP88, NUP62, and NUP153, interact with the capsid<sup>207</sup>. This evidence led to the conclusion that HIV uncoating occurs at the nuclear envelope during nuclear import, none have concluded that uncoating occurs in the nucleus<sup>208,209</sup>. However, even more recent evidence suggests uncoating happens after the CA has entered the nucleus and completed reverse transcription of its genome near integration sites just before integration<sup>26-28</sup>. Disassembly of the CA appears to occur in the nucleus by completion of reverse transcription, by yet-unknown nuclear factors, or by physical damage, and it appears to occur in tight temporal and spatial association with the integration process.

Lens epithelium-derived growth factor (LEDGF/p75) is an essential cellular cofactor of HIV integration. It binds to the viral integrase and is responsible for tethering and selective integration of HIV into active transcription units of the chromatin hence contributing to subsequent proviral gene expression<sup>210</sup>. LEDGF/p75 have also been described to play a role in the regulation of HIV latency<sup>211</sup>.

### **Viral transcription**

The positive transcription elongation factor P-TEFb, comprising cyclin-dependent kinase 9 (CDK9) and cyclin T1 or T2 (CycT1/T2), is required for RNA polymerase II phosphorylation and transcription of cellular genes. P-TEFb is recruited by Tat to the HIV promoter and is necessary for viral gene transcription. In addition to its promoter, HIV relies on an enhancer region that contains two NF- $\kappa$ B binding motifs and hence both NF- $\kappa$ B and NF- $\kappa$ B transcription factors are required for HIV transcription<sup>212</sup>. Consequently, NF- $\kappa$ B activity is modulated by HIV depending on the viral cycle stage and the signaling environment<sup>213</sup>. In the early stages of infection, Nef and Env induce NF- $\kappa$ B activity to start viral transcription from the long terminal repeat (LTR) promoter<sup>214</sup>. NF- $\kappa$ B was also shown to promote HIV latency during late stages of infection<sup>215</sup>.

### **Virus budding**

HIV Gag protein assembles in the plasma membrane of infected cells for viral particle formation. Gag polyprotein selectively recruits phosphoinositides, such as phosphatidylinositol 4,5-bisphosphate lipid [PI(4,5)P<sub>2</sub>], and cholesterol which serve as the viral budding membrane nanoplatfom<sup>216</sup>.

The endosomal sorting complex required for transport (ESCRT) participates in HIV budding<sup>217</sup>. The ESCRT machinery is indispensable for the fission of the budding viral particle from the cell surface. Gag has been found to hijack the ESCRT machinery by recruiting the tumor susceptibility gene 101 (TSG101), a member of the ESCRT-I machinery, and ALG2-interacting protein X (ALIX), part of the ESCRT-II and III<sup>216</sup>.



## Restriction factors

Although HIV hijacks the host cell and takes advantage of many cellular proteins and pathways, it has become clear that the virus had to adapt to an unfriendly environment, characterized by specific antiviral factors that may inhibit HIV at various steps of its life cycle but are largely counteracted by the viral accessory proteins. A number of expressed genes suppress viral replication in a cell-specific manner. These are referred to as restriction factors (RFs) and/or intrinsic resistance factors, and they act as a very early line of defense against infection either alongside or even before innate antiviral responses. RFs are generally IFN inducible but can also be constitutively expressed in certain cell types, which guarantees rapidity of action and confers a potent and early restriction to pathogens. The primary function of restriction factors is to directly inhibit viral replication, but their privileged interaction with retroviruses allows them to further contribute to immune detection of pathogens in some cases (Figure 7).

### Restriction factors that interfere with viral entry

Serine incorporator proteins (SERINCs) and INF-induced transmembrane proteins (IFITMs) are potent lentiviral restriction factors that inhibit infection by blocking viral entry into cells. Studies on Nef function led to the identification of SERINC3 and 5 as antiviral factors. Initially, it was discovered that the HIV Nef boosts the infectivity of HIV particles owing to an unknown role in the viral producer cell<sup>218</sup>. Two separate investigations were able to uncover these novel restriction factors<sup>219,220</sup>. SERINC5 inhibits HIV replication when the virus does not encode Nef<sup>219,220</sup>. SERINC3 proteins also inhibit HIV, but its effect is moderate compared to SERINC5<sup>219</sup>. Unlike many other restriction factors whose expression is regulated by IFNs, SERINC5 expression is independent of IFN-induced signaling<sup>219,220</sup>. In humans, SERINC5 is ubiquitously expressed across tissues and its expression is induced during monocyte differentiation to the myeloid lineage<sup>221</sup>. SERINC proteins were initially identified as carrier proteins for incorporating serines into membrane lipids. However, knock-down of SERINC1 in some immune cells had no effect on serine lipid composition or function<sup>222</sup>. Furthermore, SERINC5 had no effect on the lipid composition of HIV particles<sup>223</sup>. Recent work show that SERINC5 induced conformational changes in HIV Env protein that resulted in its functional inactivation<sup>224</sup>. Generally, SERINC5 demonstrates antiviral effects against Nef-depleted HIV viruses only when it is incorporated into viral particles<sup>219,220</sup>. The restriction action of SERINC proteins occurs mostly during virus entry, during the stage of viral and plasma membrane fusion. However, HIV Nef blocks SERINC5 incorporation into budding virions by inducing SERINC5 downregulation from the plasma membrane<sup>225</sup>. The amount of SERINC5 mRNA was found to be lower in PLWH compared to HIV-negative individuals<sup>226</sup>.

Proteins of the IFITM family (in particular, IFITM1, 2 and 3) interfere with the virus entry by insertion into the Env of nascent virions, thereby impairing cell fusion and viral spreading<sup>227</sup>. They are present in most cells of the body at a basal level, but their expression can be strongly induced after stimulation by IFN by binding to an ISRE (IFN stimulated responsive element) present in the promoter of the corresponding genes. IFITM2 and 3 are predominantly found in different endosomal compartments. IFITM1, on the other hand, is predominantly expressed at the plasma membrane since it lacks a trafficking sequence<sup>228</sup>. These proteins are effective against a wide range of enveloped viruses<sup>229</sup>. Although the original study identifying IFITMs as restriction factors did not demonstrate HIV inhibition, Lu *et al.* showed that all three IFITMs inhibit HIV replication<sup>227</sup>.

It is thought that IFITMs inhibit the fusion process during virus entry. The antiviral effects of IFITMs have been attributed to virion incorporation during assembly or inhibition of processing of the HIV envelope glycoprotein, with little consensus on their mechanism and sites of action<sup>230,231</sup>. Inhibition of the fusion step prevents the content of viral particles from being released onto the cytosol. IFITM3 is counteracted by a murine retrovirus accessory protein glycoGag, which was previously shown to antagonize the antiviral activity of SERINC proteins as well<sup>232</sup>.

### **Restriction factors that interfere with reverse transcription**

Sterile A Motif and Histidine Aspartate domain-containing protein 1 (SAMHD1) is a deoxynucleotide triphosphohydrolase that reduces the pool of cellular dNTPs, hence compromising HIV reverse transcription<sup>233</sup>. SAMHD1 hydrolyzes all four dNTPs to deoxynucleosides (dN) and inorganic triphosphate (PPPi), thereby regulating the cytosolic dNTP pool. As a result, SAMHD1 inhibits proviral DNA synthesis and HIV replication in resting T cells and myeloid cells<sup>234</sup>. Along with its dNTPase activity, SAMHD1 also has an RNase activity that might potentially contribute to SAMHD1-mediated viral restriction, by targeting viral RNA for degradation prior to reverse transcription<sup>235</sup>.

Research from our team also showed the importance of p21 in regulating the size of the intracellular dNTP pool<sup>236</sup>. p21 suppresses reverse transcription by preventing SAMHD1 phosphorylation, thus promoting SAMHD1 dNTPase antiviral activity<sup>237</sup>. In line with the relationship between cell cycling and HIV permissibility, a study showed that in the G0 phase, macrophages express p21 together with high levels of SAMHD1, thereby resulting in a highly restricted state for virus replication. A switch to G1 has been associated with the downregulation of p21, increased expression of cyclin-dependent kinase 1 (CDK1), and inactivation of SAMHD1, leading to increased dNTP levels and high virus replication<sup>238</sup>. Recent research reports a new IFN-mediated antiviral activity of SAMHD1 against RNA viruses, through downregulation of lipid synthesis<sup>239</sup>.

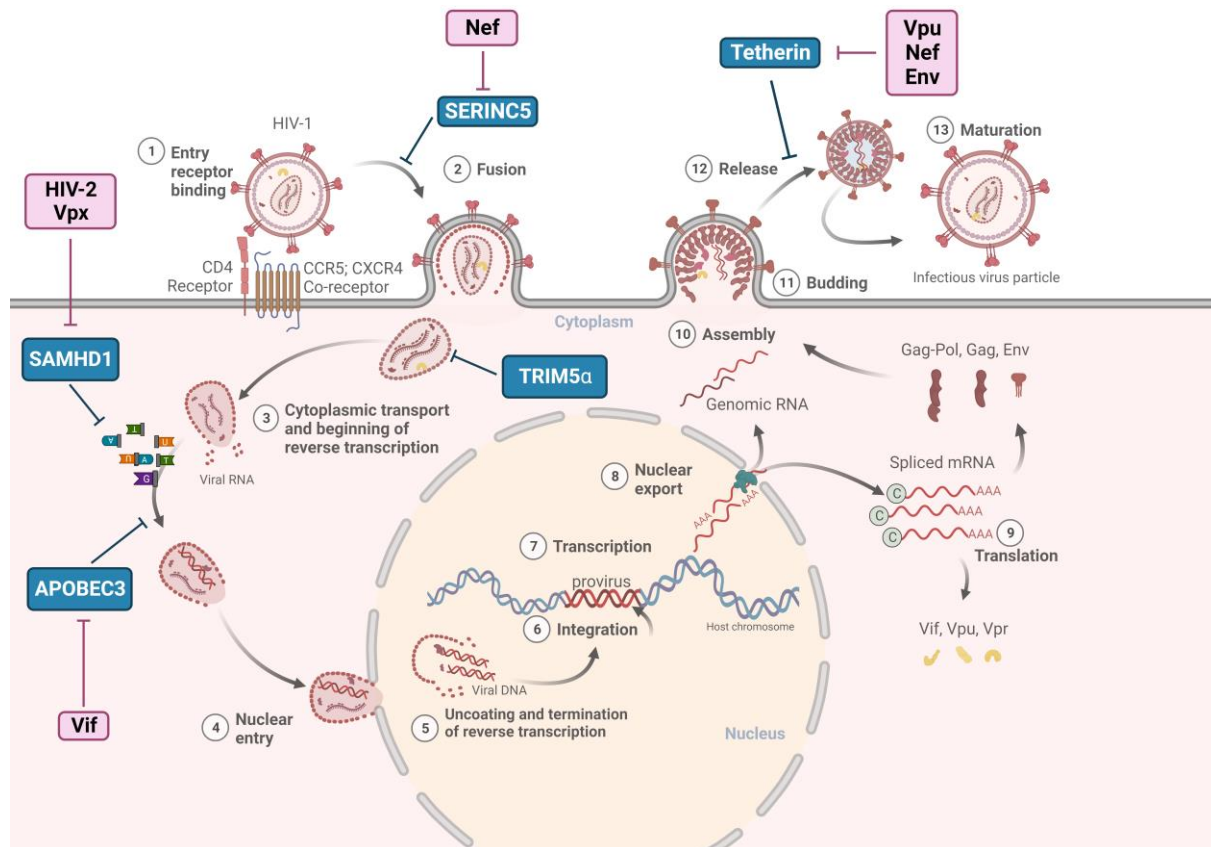
Human cells can express up to seven distinct apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3 (APOBEC3) enzymes (A/B/C/D/F/G/H), as well as more distantly related family members APOBEC1, AID, APOBEC2, and APOBEC4. During HIV infection, APOBEC3G (A3G) and other APOBEC family members, such as APOBEC3F (A3F), are sequestered within budding virions. A3G and A3F catalyze cytosine-to-uracil deamination in newly synthesized single-stranded viral DNA in freshly infected cells during reverse transcription of viral RNA. As a result, there is a high frequency of G-to-A hypermutations in proviral DNA, resulting in AA substitutions and premature STOP codons. Transcriptional activation of A3G-edited provirus results in the production of defective viral proteins and non-functional viral particles, both of which severely impede HIV spread<sup>240</sup>. Although the capacity to produce G-to-A mutations was assumed to be their principal mechanism of restriction, A3G and A3F been found to also have deaminase-independent anti-viral activities<sup>241</sup>. The HIV vif protein counteracts A3G<sup>242</sup>. Vif binding mediates proteasomal degradation of A3G as it can also downregulate the translation of A3G<sup>243</sup>.

Rhesus macaques' tripartite motif (TRIM) protein 5  $\alpha$  (rhTRIM5 $\alpha$ ) has been shown to efficiently block HIV replication, while human TRIM5 $\alpha$  (huTRIM5 $\alpha$ ) was found to be less active<sup>244</sup>. TRIM5 $\alpha$  binds directly to retroviral capsid, causing HIV to uncoat prematurely and hampering reverse transcription and genome translocation to the nucleus, hence preventing retroviral integration<sup>244,245</sup>. It was suggested that TRIM5 $\alpha$  proteins are poor inhibitors of

retroviruses that are found naturally in the same host species, but are quite often active against retroviruses that are found in other species. As such, TRIM5 $\alpha$  can impose a quite formidable barrier to cross-species transmission of primate lentiviruses<sup>246</sup>. Despite being less efficient than rhTRIM5 $\alpha$ , huTRIM5 $\alpha$  was shown to recognize HIV capsid and mediate its destabilization and premature disassembly, which disrupts the reverse transcription of the viral genome<sup>247</sup>.

**Restriction factors that interfere with virus assembly and budding**

Tetherin, also referred to as Bone Marrow Stromal Cell Antigen 2 (BST-2), attaches budding viral particles on the surface of infected cells and impairs the detachment of diverse families of enveloped viruses from infected cells<sup>248</sup>. HIV Vpu, HIV-2 Env and SIV Nef antagonize Tetherin activity<sup>249</sup>. All three viral counteracting factors redirect tetherin away from the cell surface and sequester it in a perinuclear compartment, which blocks the activity of this cellular restriction factor. Vpu also induces tetherin degradation, indicating that it employs more than one way to overcome tetherin restriction.



**Figure 7. Host restriction factors and lentiviral accessory proteins counteract them in the context of HIV viral replication.**

*SERINC5* is incorporated into HIV-1 particles and interferes with the entry and fusion steps. After virus entry, *SAMHD1* and *APOBEC3* interfere with reverse transcription (*SAMHD1*) or modify reverse-transcribed viral DNA (*APOBEC3*). *Tetherin* acts late in the replication cycle to prevent virion release. *Nef* counteracts *SERINC5* by promoting its proteasomal degradation. *Vpx* relieves the *SAMHD1*-mediated block to reverse transcription. *Vif* counteracts *APOBEC3*-driven mutagenesis of the viral genome, by preventing its packaging into virions. *Vpu* prevents *tetherin* from binding to the virus at the plasma membrane. HIV-2 and SIVs lacking *Vpu* use *Env* and *Nef*, respectively, to counteract *tetherin* by sequestering it intracellularly.

### **Role of restriction factors in the immune response**

Although restriction factors' primary role is to prevent viral replication, their direct contact with viruses enables them to also take part in the orchestration of the immune response. SERINC5 was found to play a role in innate sensing and signaling. SERINC3 and SERINC5 both increase the production of type I IFNs and the expression of nuclear NF- $\kappa$ B, which also contributes to the antiviral response<sup>250</sup>. It was also shown that IFN-I increases the surface levels of endogenous SERINC5, relocating the internal SERINC5 and stabilizing it on the plasma membrane. Furthermore, targeting IFN-I production through Jak/STAT inhibition resulted in a decrease of SERINC5 levels<sup>251</sup>. These findings implicate SERINC5 in intracellular immune signaling pathways in addition to its primary antiviral role linked with the plasma membrane.

IFITM3 deficiency has been associated to an impairment of cellular immunity most likely due to an imbalanced release of cytokines that cause lymphopenia including NK cell death and T cells depletion<sup>252,253</sup>. These findings suggest that in addition to blocking virion entry through the plasma or endocytic vesicle membrane, IFITMs restrict viruses by modulating the production of cytokines, which in turn act on various immune cells.

SAMHD1 is thought to be a negative modulator of the IFN-I inflammatory response<sup>254–256</sup>. While HIV-2 encodes the SAMHD1 antagonist Vpx, HIV, which is more pathogenic, does not. It was hypothesized that HIV-1 lacks a countermeasure against SAMHD1 because it is beneficial for the course of the infection. SAMHD1 limits HIV-1 cDNA synthesis in myeloid cells, limiting cGAS/STING-dependent responses to HIV.

Similarly, A3G appears to play a role beyond innate immunity and modulate adaptive immunity. Borzooee *et al.* and others showed that the impact of A3G-induced mutations on the HIV genome extends beyond innate immunity to adaptive immunity, by generating cytotoxic T cell (CTL) escape mutations<sup>257</sup>. A3G has been hypothesized to promote CTL escape in two ways: i) by directly mutating CTL epitopes or by ii) inducing mutations outside epitopes, which influence peptide degradation and HLA presentation of wild-type CTL epitopes<sup>258–260</sup>.

When infected with murine or simian retroviruses, human cells expressing TRIM5 $\alpha$  can act as an innate immune sensor upon recognition of the viral trigger, promote immune signaling and a cytokine response<sup>261</sup>. Interestingly, in line with their primary function as detectors of pathogens, TRIM5 $\alpha$  restriction-activity is minimized to promote its immune sensing-activity in DCs<sup>262</sup>. Finally, tetherin also acts like an immune sensor for HIV infection, activates NF- $\kappa$ B and initiates an inflammatory cytokine response<sup>263</sup>.

APOBEC3G, SAMHD1, Tetherin, and TRIM5 $\alpha$  and numerous other restriction factors have the capacity to promote cellular resistance against HIV while enhancing antiviral innate and adaptive immune responses, underscoring the multifunctional aspect of these proteins<sup>264</sup>.

### **HIV accessory proteins against restriction factors**

The biology of restriction factors is underpinned by the basic idea that HIV typically avoids their strong inhibitory effects in human cells, enabling virus replication to proceed effectively. The accessory proteins Vif, Nef, Vpu, and Vpr of HIV are typically involved in restriction factors evasion. In fact, it appears that acquiring these viral genes was mostly prompted by the need to overcome intrinsic resistance. Aside from evasion of restriction factors, the Vpu and Nef proteins influence the expression and localization of various host proteins involved in HIV replication. Vpu and Nef both decrease cell-surface expression of the key entry receptor CD4,

and MHC class I molecules, while Nef promotes T cell activation as well as HIV particle infectivity<sup>265</sup>.

Altogether, viruses have evolved sophisticated strategies to evade or directly counteract many restriction factors, driving the constant evolutionary arms race between the host and the virus<sup>265</sup>.

## Cellular metabolism

Metabolism defines the overall process by which living cells acquire and utilize the energy and intermediates required to sustain life, carry out their function, and proliferate. Anabolism, or the synthesis of complex macromolecules by the use of cellular energy, and catabolism, or their breakdown to produce needed energy, are two different types of chemical processes that constitute cellular metabolism. These two processes ultimately provide cells with required energy and metabolic intermediates, through a network of interconnected complex cellular pathways. Over the past few decades, cellular metabolism has gained a particular interest in infectious diseases and other pathologies of the immune system, giving rise to the field of immunometabolism. Recent research has demonstrated that the metabolism of T cells is very dynamic, significantly affects the regulation of T lymphocyte immunity and when altered it can modulate T cell functions<sup>266-269</sup>. In fact, it is currently acknowledged that metabolism and nutrients uptake are critical regulators of T cell activation and specialization and that nutrients act as "signal 4" to license T cell immunity by interplaying with TCR, costimulatory, and cytokine signals ("signals 1, 2, and 3")<sup>270</sup>.

Although cellular metabolism is known to be altered by viral infections for more than 50 years now, the mechanisms and consequences of virus-induced metabolic reprogramming have only begun to be studied in detail over the past decade. Viruses depend heavily on the host cell machinery to spread, therefore, it should come without surprise that viruses adopted mechanisms to promote anabolic processes for the generation of macromolecules needed for virion replication and assembly<sup>271-273</sup>. Furthermore, some viruses have evolved to have specific tropisms based on other cellular factors beyond the expression of certain receptors or co-receptors. Cellular metabolism may count as one cellular tropism determinant since not all cells have the metabolic environment that could promote viral dissemination.

As detailed in the reservoir section, not all CD4+ T cells exhibit the same susceptibility to HIV infection. For instance, resting CD4+ T cells are highly resistant to productive HIV infection, until they are activated and become susceptible. Similarly to the activation status, differentiation profile of the cell can influence its susceptibility to infection, which is demonstrated by the high resistance of naïve CD4+ T cells to HIV compared to advanced differentiation states. The distinct HIV susceptibility in CD4+ T cells depending on states of activation or differentiation has been suggested to be influenced by a number of factors such as the responsiveness to stimulation, the rigidity of the cortical actin layer, the relative expression of restriction factors, or the availability of intracellular deoxynucleoside triphosphates. Interestingly, recent data suggests that the metabolic program of CD4+ T cells may also play a critical role in regulating this hierarchy of infection, giving that both activation and differentiation are governed by adaptations in cellular metabolism.

The metabolism of key nutrients such as glucose, glutamine, other amino acids, and lipids fulfills the requirements of proliferating and differentiating CD4+ T cells. Aerobic glycolysis, pentose phosphate pathway, tricarboxylic acid cycle that fuels oxidative phosphorylation and fatty acid

$\beta$ -oxidation (FAO) are the main metabolic pathways that have been described to support T cell development, differentiation, activation and function (**Figure 8**).

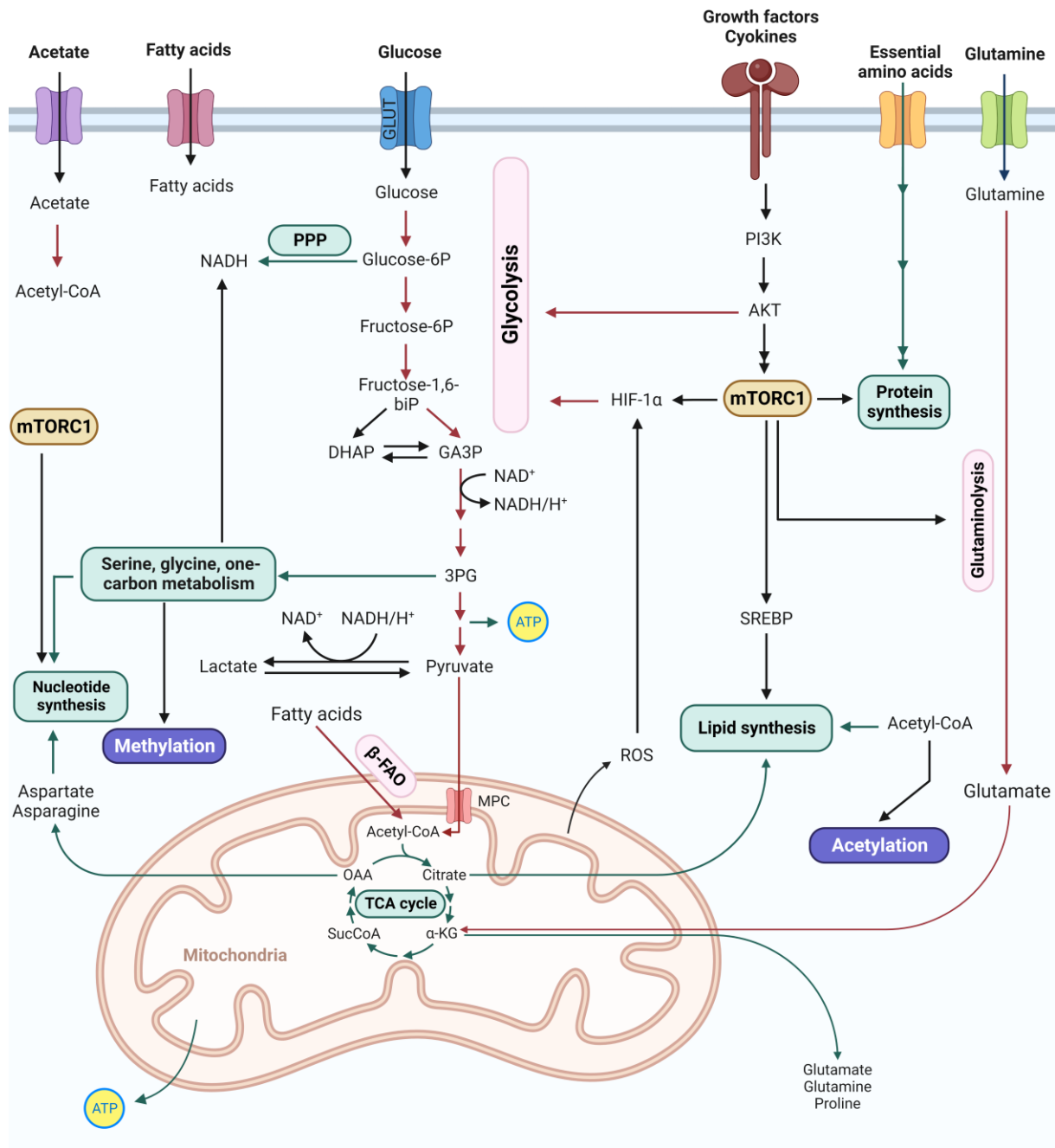
## Glycolysis

The glycolytic metabolic pathway (glycolysis) begins with the uptake of extracellular glucose from the environment surrounding the cell. Generally, T cells intake glucose through glucose transporter 1 (GLUT1) on the membrane and after phosphorylation by hexokinase (HK), one molecule of glucose is converted to two molecules of pyruvate, along with numerous other products (**Figure 8**). Then the metabolic pathway bifurcates to meet the needs of different cell functions. To maintain glycolytic flux, cells often reduce pyruvate to lactate to recycle NADH and maintain nicotinamide adenine dinucleotide (NAD<sup>+</sup>) levels. This creates a favorable redox environment that in return supports subsequent rounds of glycolysis. The generation of NADH through the reduction of NAD<sup>+</sup>, is useful for numerous enzymes that use NADH as a cofactor. Glycolytic metabolism is a relatively inefficient pathway for the generation of cellular ATP, netting only two molecules of ATP per unit of glucose. However, this process is fast and has a key role in providing biosynthetic intermediates that can fuel subsidiary pathways and the synthesis of ribose for nucleotides (glucose-6-phosphate into pentose phosphate), amino acids (3-phosphoglycerate into the serine biosynthetic pathway) and fatty acids (pyruvate into the tricarboxylic acid (TCA) cycle for citrate). The synthesis of these cellular component is important for the activated cell, but also necessary for the production of daughter cells through mitosis and mounting an immune response quickly after stimulation. In addition, faster ATP generation through aerobic glycolysis can also ensure the energy supply despite the inefficiency. Many pro-growth signaling pathways, including the phosphatidylinositol 3-phosphate kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways, promote cellular use of glycolytic metabolism. For these reasons, glycolysis is frequently observed as having a dominant and essential role in the metabolism of rapidly proliferating cells.

## TCA/OXPHOS

Alternatively, the mitochondrial tricarboxylic acid cycle (TCA cycle, also known as the citric acid cycle or Krebs cycle) occurs in the matrix of the mitochondrion and is a major metabolic pathway that is thought to be used in most quiescent or non-proliferative cell settings (**Figure 8**). Although some quiescent stem cells primarily use glycolysis, the TCA cycle and oxidative phosphorylation are a highly efficient mode of ATP generation used by cells whose primary requirements are energy and longevity.

Through OXPHOS, glucose-derived pyruvate or fatty acids can be oxidized to acetyl coenzyme A (acetyl-CoA) that join the TCA cycle by aldol condensation with oxaloacetate to form citrate. Glutamate is also a critical fuel for the TCA cycle through its direct conversion into the TCA intermediate  $\alpha$ -ketoglutarate ( $\alpha$ -KG). The TCA cycle also generates reducing equivalents (NADH and FADH<sub>2</sub>) necessary for the transfer of electrons to the electron transport chain (ETC) to support OXPHOS and highly efficient mitochondrial ATP generation. Growth signals can promote the diversion of TCA intermediates for production of amino acids and lipids (termed cataplerosis), although this requires increased nutrient diversion to the TCA cycle (termed anaplerosis).



**Figure 8. Intersection of signaling and metabolism. Growth factors and cytokine signaling can control cellular metabolism.**

Especially relevant is *mTORC1* for its role in promoting anabolic metabolism. Signaling controls metabolism through the induction of *mTOR* among other proteins that ensure an adapted response to the received stimuli through the orchestration of multiple metabolic adaptations. Such proteins can also induce the transcriptional expression of metabolic genes. Metabolism controls signaling through regulating reactive oxygen species (ROS), acetylation, and methylation 3-PG, 3-phosphoglycerate;  $\alpha$ -KG,  $\alpha$ -ketoglutarate; OAA : oxaloacetate; SucCoA : succinyl CoA; succinate-CoA; ATP, adenosine 5'-triphosphate; Glucose-6P, glucose-6-phosphate; *mTORC1*, *mTOR* complex 1; HIF-1 : hypoxia-inducible factor-1; PPP, pentose phosphate pathway; ROS, reactive oxygen species; PPP : pentose phosphate pathway; NAD/NADH : nicotinamide adenine dinucleotide oxidized/reduced form; MPC : mitochondrial pyruvate carrier;  $\beta$ -FAO : fatty acid  $\beta$ -oxidation; DHAP : dihydroxyacetone phosphate; GA3P : glyceraldehyde-3-phosphate.

## Fatty acid $\beta$ -oxidation

The fatty acid  $\beta$ -oxidation (FAO) pathway allows for the mitochondrial conversion of fatty acids into numerous products that the cell can further use to generate energy, including acetyl-CoA, NADH and FADH<sub>2</sub> (**Figure 8**). Short-chain fatty acid (six or less carbons) diffuse into mitochondria passively, while medium-chain and long-chain fatty acid must first be conjugated to carnitine via carnitine palmitoyl transferase I (CPT1). Carnitine conjugated long-chain fatty acid is then shuttled into the mitochondria where it is converted back to a fatty acid acyl-CoA through the removal of carnitine by carnitine palmitoyl transferase II (CPT2). In the mitochondria,  $\beta$ -oxidation of the fatty acid acyl-CoA commences, yielding large amounts of acetyl-CoA, NADH and FADH<sub>2</sub> that are further used in the TCA cycle and the electron transport chain to generate ATP. CPT1A is inhibited by the lipid synthesis intermediate malonyl-CoA, thus preventing lipid oxidation when cells are actively synthesizing lipids. Overall, FAO can allow the production of tremendous amounts of ATP, with the complete  $\beta$ -oxidation of a single palmitate molecule (a major fatty acid in mammalian cells) eventually having the potential to yield over 100 ATP molecules.

## The pentose phosphate pathway (PPP)

PPP takes place in the cytosol and is a major pathway that supports cell proliferation and survival. PPP uses intermediates from the glycolytic pathway to allow the production of nucleotide and AA precursors (**Figure 8**). A second key function of the PPP is the generation of reducing equivalents of nicotinamide adenine dinucleotide phosphate NADPH, which has an important role in the maintenance of a favorable cellular redox environment and is also required for fatty acid synthesis.

## Fatty acid synthesis

The fatty acid synthesis (FAS) pathway allows cells to generate lipids that are necessary for cellular growth and proliferation. FAS uses products derived from several other metabolic pathways, notably glycolysis, the TCA cycle and PPP (**Figure 8**). For the synthesis of straight-chain fatty acids, TCA cycle-derived citrate may be exported from the mitochondria into the cytosol via the citrate carrier, where ATP citrate lyase converts it into acetyl-CoA, along with oxaloacetate. The acetyl-CoA derived from this process may then be carboxylated by the acetyl CoA carboxylase (ACC) to yield malonyl-CoA. Subsequently, the fatty acid synthetase (FASN) acts in an NADPH-dependent manner to elongate the nascent fatty acid chain until products such as palmitic acid are synthesized. Fatty acids with alternative chain lengths may be synthesized using palmitic acid as a substrate for elongation. Furthermore, fatty acids may be condensed with glycolysis-derived glycerol to yield many possible combinations of triacylglycerols and phospholipids, which are key components of many cellular structures.

The activity of the FAS pathway is closely coupled to mammalian target of rapamycin (mTOR) signalling, which has been shown to promote FAS through regulation of many of the key enzymes responsible for de novo lipid synthesis, including sterol regulatory element binding protein (SREBP) as well as ACC and FASN, which are both induced by SREBP.



## Amino acid metabolic pathways

Aside from their well-known role as building blocks for cell proliferation, amino acids can act as precursors for the *de novo* synthesis of branched-chain fatty acids. They play an important role in controlling immune responses. Individual amino acids may play more specific roles in metabolic pathways. For instance, branched-chain amino acids such as valine and leucine serve as substrates for branched-chain fatty acid synthesis. Glutamine and aspartate are required for *de novo* purine and pyrimidine synthesis. Glutamine is the most prevalent AA in physiological fluids and cell culture media, providing energy and nitrogen to cellular metabolism. Glutamine enters the TCA cycle via the action of glutaminase (GLS) and glutamate dehydrogenase (GDH) and serves as a significant alternate carbon source for virus-infected cells<sup>272,274,275</sup>.

In addition, glutamine may be used as an alternative input for the TCA cycle where it can be used to support ATP production or, alternatively, as a source of citrate for fatty acid synthesis. Additional amino acids, including arginine and tryptophan, are metabolized through various metabolic pathways to support cellular proliferation and anabolic growth. amino acids are intimately linked to important anabolic cellular signalling pathways, most notably the mTOR pathway and nucleotide synthesis.

## Immunometabolism

Cellular metabolism does much more than simply provide cells with energy in the form of ATP and/or biomass. It has been shown that different metabolites and metabolic fluxes can modulate cell signaling pathways and post-translational modifications<sup>276</sup>. Metabolism has been shown to regulate histone acetylation and influence gene expression in immune cells<sup>277</sup>. Metabolic enzymes can also serve as cellular or pathogen-derived RNA-binding proteins and have been reported to exhibit ‘moonlighting’ functions<sup>278–280</sup>. Finally, many metabolites can act directly as signaling molecules to influence either pro-inflammatory or anti-inflammatory outcomes<sup>276,281–283</sup>.

Metabolic reprogramming is essential for immune cells activation, differentiation, function and fate<sup>284,285</sup>. This finding explains the unique metabolic profiles for different subsets and different phenotypes of immune cells<sup>286,287</sup>. An immune response is dynamic and consists of phases of cellular activation, inflammation and resolution. It is now clear that immune cell activity can be regulated, in part, by metabolic rewiring driven by cell-intrinsic factors and extracellular nutrient availability. This was best demonstrated in T cells, where for example, naïve, effector and memory T cells adapt different bioenergetics phenotypes that correlate and support their differentiated states. The energy and biosynthetic needs of quiescent T cells are facilitated by catabolic metabolism of glucose and amino acids, whereas the egress from quiescence is prompted by alterations in catabolic and anabolic metabolism<sup>288</sup>. Indeed, the metabolic characteristics obtained after the transition from quiescence allow for higher intake of external resources for biosynthesis and energy generation<sup>289</sup>.

Effector T cells rely heavily on glycolysis compared to OXPHOS, but switch to FAO and OXPHOS-dominant metabolism when they transition to a memory resting state. For years, the idea that cells rely either on OXPHOS or on glycolysis was propagated in the field of immunometabolism, but now we understand that this was an oversimplified interpretation of a much more complex system. The reality is that each metabolic pathway provides unique inputs

into the overall function of the cell, despite changes in the degree to which glycolysis and OXPHOS are used<sup>290,291</sup>.

In the past years, there have been major technological advances in measuring metabolites and metabolism. Advances in single-cell technologies in particular are pushing the boundaries of what is possible for analyzing metabolism within heterogeneous cell populations. These recent advances and discoveries made immunometabolism an emerging and crucial research topic in immunology, with promising therapeutic strategies.

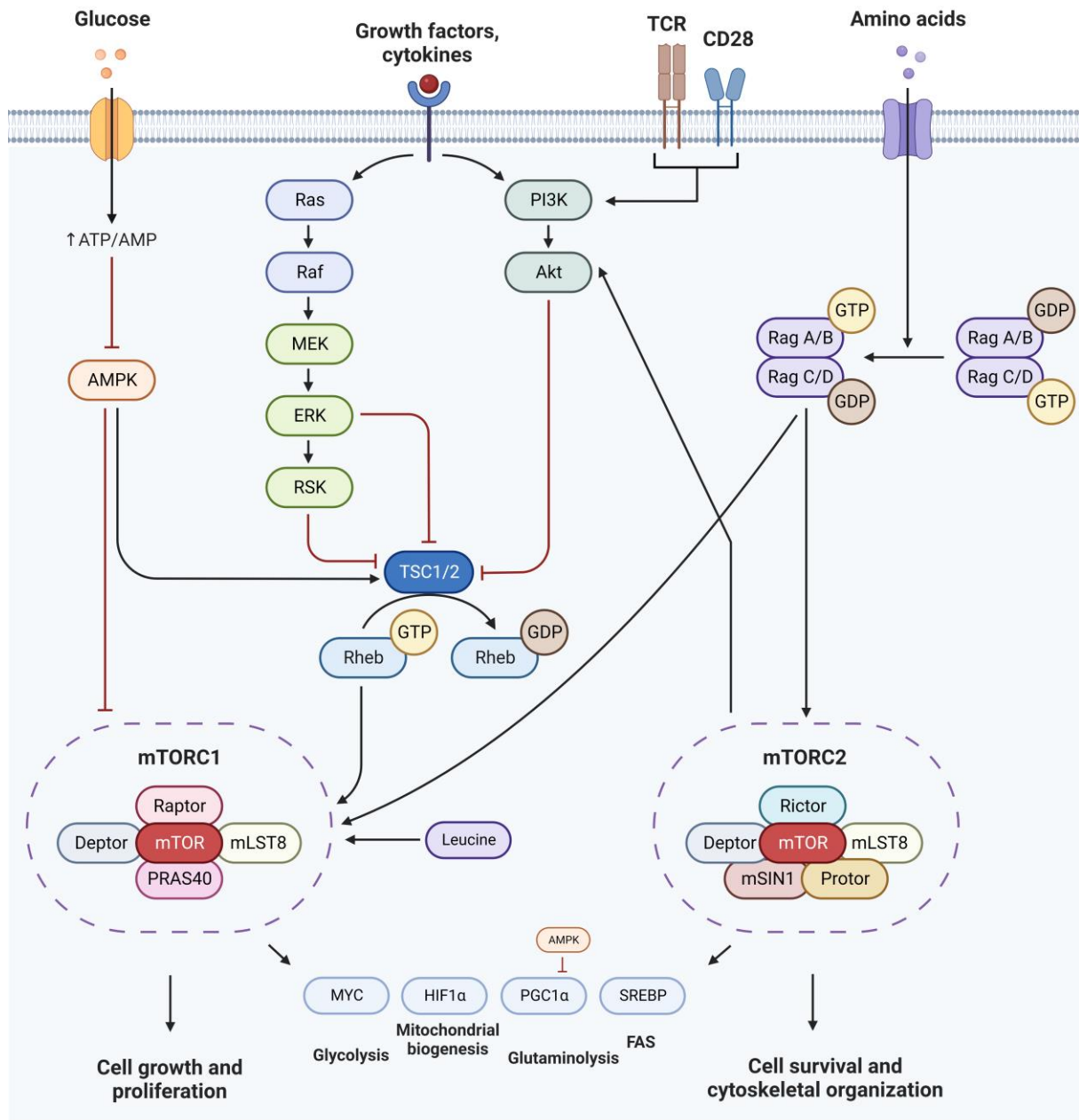
Next, some examples of the interplay between metabolic pathways and immune cell function are discussed.

## **Mechanistic target of rapamycin (mTOR)**

The mechanistic target of rapamycin (mTOR)<sup>292</sup> is the master integrator of the external availability of nutrients, energy and growth factors, but also the master regulator of the different responses to these informations. Depending on the information integrated into the cell, mTOR coordinates the synthesis or breakdown of new cellular components. mTOR is one of the key regulators of T cell quiescence exit<sup>288</sup>. It is found at the crossroads of several important signaling pathways and is essential in orchestrating the cellular and organismal physiology of all eukaryotes. mTOR regulates cellular metabolism by controlling the translation and stability of key metabolic transcription factors (**Figure 9**). mTOR is a serine/threonine protein kinase that belongs to the PI3K-related protein kinases family<sup>293</sup>. For it to induce its downstream signaling pathways, this protein kinase needs to form complexes with other cellular component. mTOR is the catalytic subunit of two distinct complexes known as mTOR complex 1 (mTORC1) and mTORC2.

Their accessory proteins and varied rapamycin (a microbial macrolide that inhibits mTOR signaling and is at the origin of its discovery) sensitivity, as well as their distinct substrates and functions differentiate these complexes. mTORC1 is defined by a regulatory-associated protein of mTOR (RAPTOR), while mTORC2 is defined by rapamycin-insensitive companion of mTOR (RICTOR)<sup>294</sup>. mTORC1 phosphorylates substrates that promote protein, lipid, nucleotide, and ATP synthesis while inhibiting autophagic degradation of cellular components<sup>295</sup>. mTORC2 also helps to quiescence egress. This kinase activates the oncogene Akt, a key early effector in the PI3K pathway, where it modulates insulin response and promotes proliferation<sup>296</sup>. Akt and other related protein kinases directly phosphorylate the forkhead-box FOXO1/3a transcription factors, resulting in FOXO proteins exclusion from the nucleus and transcriptional activity repression<sup>297</sup>. Moreover, Akt facilitates the transfer of GLUT1 to the cell membrane and HK to mitochondria<sup>298-300</sup>. Akt also phosphorylates NAD kinase and catalyzes the synthesis of NADP<sup>+</sup>, which is the primary cofactor of synthetic reductive metabolism<sup>301</sup>. Akt also regulates the activity of glycogen synthase kinase 3b (GSK3b), which suppresses apoptosis and modulates glucose homeostasis.

mTOR-deficient T cells fail to engage in metabolic reprogramming upon activation and do not proliferate or gain effector function<sup>302</sup>. The role of mTOR in T cell metabolism is selective, as treatment of CD8<sup>+</sup> T cells with the mTOR inhibitor rapamycin reduces the abundance of metabolic proteins, such as glucose transporters and enzymes involved in glycolysis and cholesterol metabolism, but not others involved in mitochondrial OXPHOS and glutamine metabolism<sup>303</sup>.



**Figure 9. Overview of PI3K/mTOR signaling pathway.**

The catalytic subunit of PI3K is activated as a result of stimulation of certain surface receptors (growth factor receptors, cytokines, etc.). When PI3K is activated, it phosphorylates the secondary messenger phosphatidylinositol-3,4,5-bisphosphate (PIP<sub>2</sub>), which then transforms to phosphatidylinositol-3,4,5-triphosphate (PIP<sub>3</sub>) (PIP<sub>3</sub>). PIP<sub>3</sub> is crucial for recruiting the protein kinase AKT to the cell surface, where it is activated/inhibited by a variety of molecules, leading to its engagement in a number of downstream signaling cascades. The key negative regulators of the process are phosphatase and tensin homologue deleted on chromosome 10 (PTEN), tuberous sclerosis protein 1 (TSC1), and TSC2, with AKT phosphorylation of TSC2 releasing the inhibitory impact on mTORC1 via the GTP-binding protein Rheb. mTORC1 activates p70S6K while inhibiting 4E-BP1, resulting in protein synthesis and cell growth.

In addition to signals downstream of the TCR, second and third signals, T cell metabolism is further regulated as a consequence of the significant metabolic changes they undergo, in a mechanism described as “*bottom-up signaling*”<sup>304</sup>. One example of this is the activation of mTOR by AA sensing. Expression AA transporters is required for T cells to become activated and differentiate into effector phenotypes. mTOR activity is boosted in response to increased abundance of leucine and arginine, which drives an mTOR-dependent metabolic reprogramming in T cells<sup>305</sup>.

Finally, AMPK acts as a counterweight to mTOR. Rising AMP levels, which indicate increased energy expenditure, activate AMPK, which stimulates ATP synthesis by phosphorylating targets in the glycolytic and FAO pathways. It also prevents further ATP consumption by blocking catabolic processes, protein translation, and mTOR activation. Under nutrient-poor situations, AMPK plays a key function in regulating metabolic flexibility of T cells. When activated in glucose-replete settings, quiescent T cells switch to aerobic glycolysis. A low-nutrient environment, on the other hand, causes metabolic reprogramming governed by AMPK activation. To maintain bioenergetic needs and viability, cells activate glutamine-dependent OXPHOS and repress mRNA translation<sup>306</sup>.

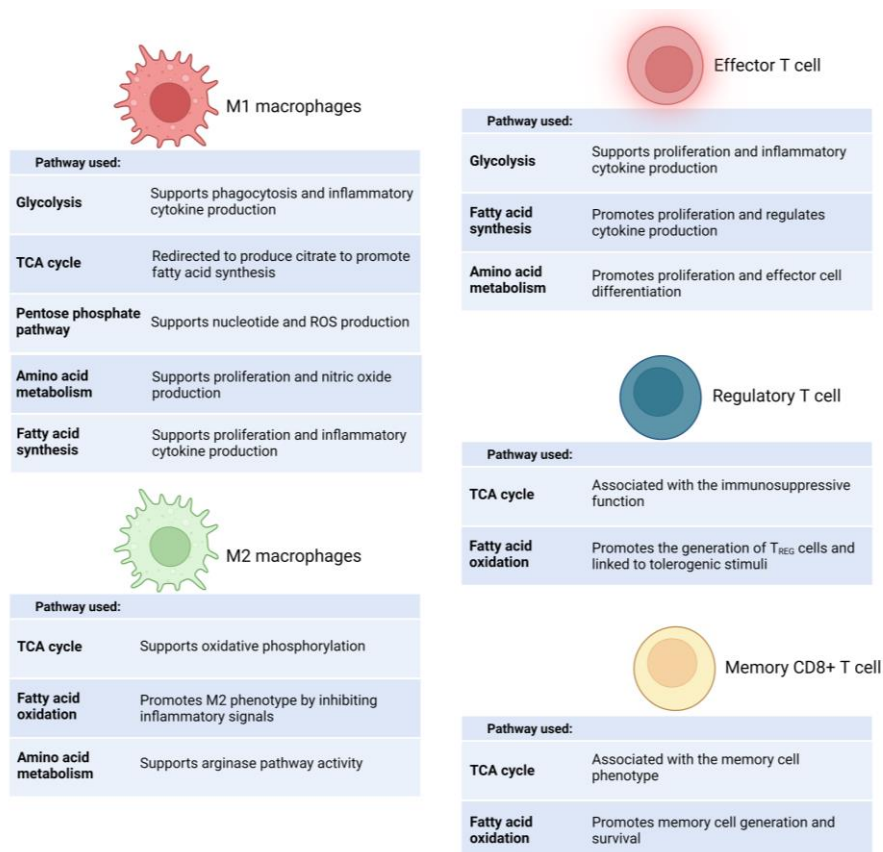
## Glycolysis

Glycolysis has been shown to be involved in a number of immune processes. T cells and macrophages rely heavily on glycolytic pathways (**Figure 10**). The use of 2-deoxyglucose (2-DG), a competitive glucose analog that is not metabolized by cells, inhibits activation and suppresses inflammation in *in vivo* models. This led to the conclusion that glycolysis is crucial for immune cell function. As mentioned above, glycolysis is not very efficient for ATP production and it was unexpected to note that immune cells, which need a vigorous amount of ATP, rely more on this glycolytic pathway instead of the TCA cycle. However, it was later realized that glycolysis is rapidly employed via the induction of the enzymes that are involved in this pathway. OXPHOS, on the other hand, requires mitochondrial biogenesis, which is a more complex and slower process. Hence, cells in need of rapid ATP and crucial biosynthetic intermediates to support rapid cell growth and/or to carry out its particular effector functions such as, phagocytosis and inflammatory cytokines production for macrophages, antigen presentation for DCs and production of cytokines for T cells switch to glycolysis<sup>307</sup>.

Resting T lymphocytes predominantly use the TCA cycle to metabolize glucose and generate NAD to drive OXPHOS. Only in anaerobic circumstances is glycolysis activated, producing high quantities of lactate while using less ATP. Most cancer cells create a considerable quantity of lactate independent of oxygen supply, and this "aerobic glycolysis" was initially noticed by Otto Warburg and is now known as the Warburg effect<sup>308</sup>. Activated T cells are one example of proliferating cells that drastically upregulate the comparatively inefficient aerobic glycolysis, turning pyruvate into lactate even with abundant oxygen, a mechanism that requires no involvement from mitochondria but results in reduced ATP production<sup>309</sup>. Enhanced glycolysis occurs in activated cells including macrophages, DCs, NK cells, effector T cells and B cells. All effector T cell subsets show an increase in glycolysis following activation, most notably T<sub>H17</sub> cells, T<sub>H1</sub> and T<sub>H2</sub> cells and activated effector CD8+ T cells. Increased glycolysis can be considered a hallmark metabolic change in most immune cells undergoing rapid activation, for instance, in response to stimulation of pattern-recognition receptors (PRRs), cytokine receptors or antigen receptors.

Another interesting role that the glycolytic pathway plays in regulating immune cells and immune response was observed in  $T_{H1}$  cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) can bind to the IFN- $\gamma$  mRNA, thus repressing its translation<sup>310</sup>. Once glycolysis is upregulated, GAPDH dissociates from *IFNG* mRNA to enter the glycolytic pathway flux, allowing the translation of IFN- $\gamma$ . Thus, active glycolysis with the engagement of GAPDH is essential for effector cytokine production including IL-2 and IFN- $\gamma$ . While lactate dehydrogenase A (LDHA) enhances histone acetylation and *ifng* transcription by acting epigenetically by raising acetyl-CoA concentrations<sup>311</sup>. Additionally, glucose restriction partially or totally decreases the synthesis of IFN- $\gamma$ , GM-CSF, and TNF- $\alpha$  in CD8+ T cells<sup>312,313</sup>.

Similarly, metabolic control may direct T cell differentiation. Studies show that inhibiting glycolysis inhibited both transcriptional and translational synthesis of IFN- $\gamma$  and  $T_{H1}$  differentiation<sup>314</sup>. The discovery that inhibiting mTOR signaling inhibits naïve CD4+ T cells from developing into pro-inflammatory effector cells while selectively activating mTORC1 or mTORC2 results in unique differentiation into  $T_{H1}$  and  $T_{H17}$  or  $T_{H2}$  cells further supports the significance of glucose metabolism<sup>315,316</sup>.



**Figure 10. Metabolic pathways preferentially utilized in different immune cell populations.**

The various immune cell subsets exhibit a reliance on distinct metabolic pathways to promote cell survival, lineage generation and function. Inflammatory macrophages use glycolysis, the tricarboxylic acid (TCA) cycle, the pentose phosphate pathway, fatty acid synthesis and amino acid metabolism to proliferate and to support the production of inflammatory cytokines. M2 macrophages, which exhibit a more tolerant phenotype, use the TCA cycle, fatty acid oxidation and arginine flux into the arginase pathway. Rapidly proliferating effector T cells, including  $T_{H1}$ ,  $T_{H17}$  and cytotoxic CD8+ T cells, use glycolysis, fatty acid synthesis and amino acid metabolism to promote proliferation and cytokine production. TREG cells use the TCA cycle and fatty acid oxidation. Similarly, memory CD8+ T cells also require the use of the TCA cycle and fatty acid oxidation to promote increased cell lifespan. ROS; reactive oxygen species (adapted from O'Neill, L., Kishton, R. & Rathmell, J. *Nat Rev Immunol.* 2016<sup>914</sup>).

Hexokinase 1 (HK1) is another glycolytic enzyme that has also been shown to have a moonlight activity by regulating NLRP3 (NOD-like receptor family, pyrin domain containing 3). The NLRP3 inflammasome is a crucial regulator of caspase 1, which generates mature IL-1 $\beta$ , as well as IL-18, and it induces cell pyroptosis. HK1 can interact with NLRP3 and enable its activation.

## **The Pentose phosphate pathway (PPP)**

The pentose phosphate pathway produces two main byproducts: the synthesis of nucleotides (which happens during cell proliferation) and NADPH. NADPH is crucial for immune cells function during immune response. It is employed by the NADPH oxidase to generate reactive oxygen species (ROS), but at the same time, it is used to produce glutathione and other antioxidants. Both generation of ROS and the antioxidant potential of NADPH is crucial for macrophages and neutrophils function during an ongoing infection. ROS helps with the clearance of pathogens and antioxidants minimize local tissue damage. DCs also rely on NADPH, couples with lipid synthesis, for the assembly of endoplasmic reticulum, which is critical for their activation and cytokine release<sup>317</sup>. PPP has been recently propose to play a key role in immune homeostasis by controlling the suppressive function of T<sub>REG</sub> cells<sup>318</sup>.

## **TCA cycle and oxidative phosphorylation**

Glycolysis and mitochondrial respiration are the major focus in immunometabolism. Both routes are utilized by effector cells, despite the fact that these latter have a propensity to switch from TCA cycle to glycolysis, as already aforementioned. After activation, both CD4+ and CD8+ T cells exhibit increased glycolysis, with CD8+ T cells being more glycolytic and better able to use glutamate, which correlates with their greater ability for growth and proliferation<sup>319,320</sup>. Although glycolysis is required for T cell activation, OXPHOS is also increased upon T cell activation<sup>267</sup>. Memory T cells usually display higher TCA cycle activity<sup>321</sup>. In general, OXPHOS is often associated with a more anti-inflammatory cell phenotype. TCA cycle has been specifically much studied in M1 and M2 macrophages that use this pathway in a distinct way. The TCA cycle is coupled to OXPHOS in M2-macrophages. As a result, UDP-GlcNAc intermediates are produced. These intermediates are required for the glycolysation of M2-macrophages receptors<sup>322</sup>. However, M1 macrophages exhibit a disrupted TCA cycle, where it is shown to be interrupted in two steps; after citrate and after succinate. The accumulated citrate is then used for the production of fatty acids, nitric oxide and prostaglandins. In addition, itaconic acid, a metabolite produced from citrate, has been demonstrated to have antibacterial effects<sup>323</sup>. This interrupted TCA cycle feature is also observed in activated DCs that require substantial membrane production for antigen presentation<sup>317</sup>. Similar to citrate, succinate also accumulates in M1 macrophages as a result of a broken TCA cycle<sup>324</sup>. Succinate interacts and inhibits prolyl hydroxylases, resulting in a stabilization of hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) and production of IL-1 $\beta$ . Furthermore, another mitochondrial metabolite, itaconate, can block succinate dehydrogenase (SDH) in LPS-activated macrophages. Itaconate and its membrane-permeable derivative dimethyl itaconate inhibit the production of inflammatory cytokines such as IL-1, IL-18, IL-6, and IL-12, hence reducing inflammation and delivering negative feedback to the host immune system<sup>325</sup>. These

findings clearly show that TCA cycle metabolites can influence cytokine production in host immunological responses.

## **Fatty acid $\beta$ -oxidation**

FAO plays an important role in regulating T cell responses. It has been shown to modulate the balance between inflammatory effector T cells and suppressive T<sub>REG</sub> cells, as well as promoting long-lived memory T cells, which are important for long-term immune protection. T<sub>REG</sub> use FAO largely when compared to effector cells such as T<sub>H1</sub>, T<sub>H2</sub> and T<sub>H17</sub>. In addition, FAO was shown to promote T<sub>REG</sub> cells while suppressing effector T cell polarization<sup>326</sup>. Consistent with these observations, additional studies revealed that T<sub>REG</sub> cells upregulate the expression of FAO-related genes, including *Cpt1a* gene, when compared to T<sub>H17</sub> for example. It is also interesting to note that during activation, effector T cells decrease FAO. Ligation of PD1 receptor on T cells resulted in increased expression of CPT1A and enhanced FAO, which is in alignment with the previously mentioned findings of how FAO impairs effector T cell activity and induces tolerance.

FAO is also important in the development and maintenance of memory CD8+ T lymphocytes. In absence of antigen or any other stimulation, memory CD8+ T cells proliferate at slow pace. Once a stimulus is encountered, these cells require FAO for a prompt immune response<sup>327</sup>. In that manner, stimulation of memory CD8+ T cells with IL-15 was shown to enhance CPT1A expression, hence boosting FAO and resulting in better cell survival<sup>328</sup>. Interestingly, despite using FAO as a major bioenergetics pathway to support their function, memory CD8+ T cells also engage FAS pathways to synthesize *de novo* fatty acids that would fuel FAO process<sup>307</sup>.

These findings suggest that FAO plays an important role in both the development of tolerogenic T<sub>REG</sub> cells and in the preservation of long-lived memory CD8+ T cells.

## **Fatty acid synthesis**

Recent studies demonstrated that deletion of acetyl-CoA carboxylase 1 (ACC1), an enzyme implicated in FAS, results in decreased efficacy and poorer recruitment of antigen-specific CD8+ T cells. Furthermore, these alterations were corrected when exogenous fatty acids were added to these ACC1-deficient cells<sup>329</sup>. In CD4+ T cells, inhibition of ACC1 showed that FAS is essential for T<sub>H17</sub> cell differentiation but not for T<sub>REG</sub> cells development and function<sup>330</sup>. A protein termed CD5 antigen-like (CD5L) expressed in a specific T helper cell type, termed “non-pathogenic” T<sub>H17</sub> – they secrete low levels of IL-17 and produce the anti-inflammatory cytokine IL-10 - was found to bind the fatty acid synthetase (FASN) to promote the production of polyunsaturated fatty acid that enhance the production of IL-10 while limiting IL-23 and IL-17 expression<sup>331</sup>. In pathogenic T<sub>H17</sub> cells, CD5L expression is low. This may be advantageous for the production of saturated fatty acid that indirectly increase the expression of the pro-inflammatory cytokines, IL-17 and IL-23 while inhibiting the expression of IL-10. This indicates that the type of secreted cytokines and hence the nature of the immune response can depend on the form of fatty acid synthesized in these T helper cells. This work exemplifies the promise of delving into immunometabolism in order to obtain new understandings regarding immune cell effector function.

Thus, in contrast to FAO, which appears to favor non-inflammatory, tolerogenic immune cell growth and activity, FAS appears to positively control the creation and function of pro-inflammatory immune cells.

IL-6 increases lipid oxidation in skeletal muscle via an AMPK-dependent mechanism<sup>332</sup>, implying a link between lipid metabolism and cytokines. Non-esterified fatty acids (NEFA) have been shown to increase cytokine production of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-10 in human trophoblasts, lending credence to the interaction between lipid metabolism and inflammation<sup>333</sup>. Furthermore, lipid receptors were found to promote JAK/STAT-mediated IFN signaling in macrophages and B lymphoma cells<sup>334,335</sup>. Similarly, FAS regulators such as ACC and lysophosphatidic acid (LPA) regulate DC activity and modify the production of cytokines such as IL-12, TNF- $\alpha$ , and IL-10<sup>336</sup>.

## **Amino acid metabolism**

Glutaminolysis is another essential metabolic process that is considerably increased following T cell activation, as evidenced by the fact that treatment of T cells with glutamine antagonist reduces T cell growth<sup>337,338</sup>. Carbon tracing experiments showed that glutamine carbons are incorporated into intermediate metabolites in the TCA cycle, which might imply that glutamine feeds mitochondrial ATP generation in activated T cells<sup>339</sup>.

Indoleamine-2,3-dioxygenase IDO, an enzyme that is responsible for the rate-limiting phase of tryptophan catabolism, has been the subject of many studies on the immunoregulatory role of tryptophan metabolism in the immune system. IDO expression has been found to be upregulated by type I IFNs. The breakdown of tryptophan, which is necessary for T cells proliferation *in vitro*, by IDO in APCs inhibits T cell activation<sup>340,341</sup>.

Arginine has been reported to affect the expression of TCR components and to increase the proliferation of T cells<sup>342,343</sup>. Furthermore, mTORC1 activity in T cells is decreased in arginine, leucine, or glutamine depleted conditions<sup>344,345</sup>. Similarly, knockdown of ASCT2 (cellular receptor for neutral amino acids such as glutamine and leucine) in T cells resulted in altered development and function of TH1 and TH17 cells, while TREG formation was not affected<sup>346</sup>. It was shown that ASCT2 deletion or reduction of glutamine levels in culture conditions caused a drop in mTORC1 activity, which coincided with the reported effector T cell defects, demonstrating once again the critical link between amino acid, particularly glutamine, levels and mTOR pathway activity in the immune system.

## **Metabolic program of immune cells**

During immune activation, epigenome remodeling, wherein the addition or removal of epigenetic marks alters chromatin accessibility, occurs. This coincides with metabolic reprogramming in newly activated immune cells. Epigenetic modifiers happen to be catalyzed by the availability of substrates derived from metabolic pathways and regulated by metabolites that could inhibit their function. For example,  $\alpha$ -KG, a product of TCA cycle, is required for histone demethylase activity, which can be inhibited by high levels of succinate, fumarate or 2-hydroxyglutarate (2HG); all three are also intermediates of the TCA cycle<sup>347</sup>. Thus, the dependence on distinct metabolic pathways can regulate the abundance of metabolites required to maintain or reprogram the epigenome, resulting in modulation of immune cell identity and function<sup>348,349</sup>.



In addition to playing a major role in epigenetic remodeling and defining T cell function, metabolic intermediates were found to influence cytokine expression by regulating the extent of histone acetylation<sup>350,351</sup> and methylation<sup>311,352,353</sup>. By modulating the intracellular pools of metabolites required for epigenetic activity, the availability of these nutrients affected the capacity to carry out epigenetic modifications, resulting in altered effector cytokine expression. It is also believed that metabolic dysfunction may influence the expression of exhaustion markers, also by regulating the epigenome of immune cells<sup>354,355</sup>. T cell response checkpoints inhibitors include PD-1 (CD279) and CTLA-4 (CD152). Intriguingly, the expression of inhibitory immune checkpoint proteins on immune cells such as macrophages and DCs can also alter their metabolic activity and result in their dysfunction. During persistent viral infection, PD-1 signaling inhibits aerobic glycolysis and creates a metabolic-impairment phenotype in viral-antigen-specific CD8+ T cells<sup>356,357</sup>. Furthermore, PD-1 activation reduces the expression of peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 (PGC1 $\alpha$ ), a transcription factor that regulates mitochondrial biogenesis. Consequently, mitochondrial biogenesis, function, and capacity to conduct oxidative phosphorylation are reduced<sup>356,358</sup>. Due to the overall drop in bioenergetics caused by PD-1 activation, T cells differentiate into an exhausted phenotype<sup>356</sup>. Furthermore, exhausted viral antigen-specific CD8+ T lymphocytes from individuals infected with hepatitis B virus, hepatitis C virus, or PLWH show a reduction in metabolic fitness<sup>359,360</sup>.

## HIV infection and immunometabolism

It is becoming increasingly clear that cellular metabolism plays a significant role in determining the vulnerability of cells to HIV; for instance, restricting glucose transport and glycolysis prevents HIV replication and viral spread from reservoirs *in vitro*<sup>361–363</sup>. Glycolysis and glutaminolysis are essential for HIV infection and replication and blockade of these processes using galactose, 2-DG, or 6-diazo-5-oxo-l-norleucine (DON) might significantly reduce viral infection<sup>361,362,364</sup>. A study showed that catalytic mTOR inhibition alters cellular metabolism to hinder the initial stages of HIV replication after entrance<sup>365</sup>, giving an explanation to how inhibiting T cell glucose transport and glycolysis impairs HIV replication. Increases in the expression of the glucose and glutamine transporters (Glut1 and ASCT2, respectively) are among the mTOR-dependent mechanisms necessary for HIV reverse transcription. Earlier reports demonstrated the importance of the stabilization of microtubules by acetylation, for the cytoplasmic transport of reverse transcription products, a process that requires an mTOR-mediated metabolic cascade<sup>366</sup>. Cellular glutamine and glycolytic products, both of which are boosted by mTOR activity, contribute to microtubules acetylation via the TCA cycle.

Reducing the dependence on glycolysis and recovering mitochondrial activity may improve CD8+ T cell antiviral function<sup>367,368</sup>. The question of whether cellular metabolism might be addressed as a novel therapeutic strategy for treatment has arisen in light of our growing understanding of the immunometabolic alterations associated with HIV infection. With the exception of *in vitro* research, attempts to verify such hypotheses are still incredibly rare because it is such a newly growing discipline.

Metformin, which reduces glucose production in the liver and is widely used for the treatment of type 2 diabetes, was tested in a pilot study to investigate its impact on the HIV reservoir in non-diabetic PLWH on ART<sup>369</sup>. This trial was prompted by reports indicating that metformin

may act as a mTOR inhibitor, along with the documented role of mTOR in regulating cell metabolism<sup>370,371</sup>, and HIV transcription<sup>372</sup>, primarily in T<sub>H17</sub> cells<sup>373</sup>. Results of this trial showed that metformin had beneficial effects in attenuating inflammation and residual HIV transcription in the colon of ART-treated PLWH via mechanisms involving a reduction in mTOR phosphorylation/activation<sup>369</sup>.

## High metabolic activity drives infection

The dynamics of CD4+ infected subtypes in HIV infection change during infection<sup>123,126,141,374</sup>. This can be due to a change in the metabolic profile of cells. Activated CD4+ T cells are thought to be the preferred target of HIV infection and recent research indicates that metabolically active cells are more susceptible to HIV infection. CD4+ T cells expressing GLUT1 had greater levels of activation marker as well as higher levels of HIV co-receptor CCR5, when compared to CD4+GLUT1- cells. In addition, the use of PI3K inhibitors and the blockade of glycolysis pathway was shown to suppress HIV infection *in vitro*<sup>363</sup>. These data led to the belief that cellular metabolic activity matches immunological phenotype, and that both may indicate vulnerability to HIV infection. However, previous work from our team revealed that although there is a link between HIV susceptibility and metabolic activity, this correlation do not necessarily match activation status<sup>362</sup>: activated but weakly glycolytic cells were extremely resistant to infection, whereas highly glycolytic T<sub>CM</sub>, but also T<sub>N</sub> cells, were susceptible to HIV infection.

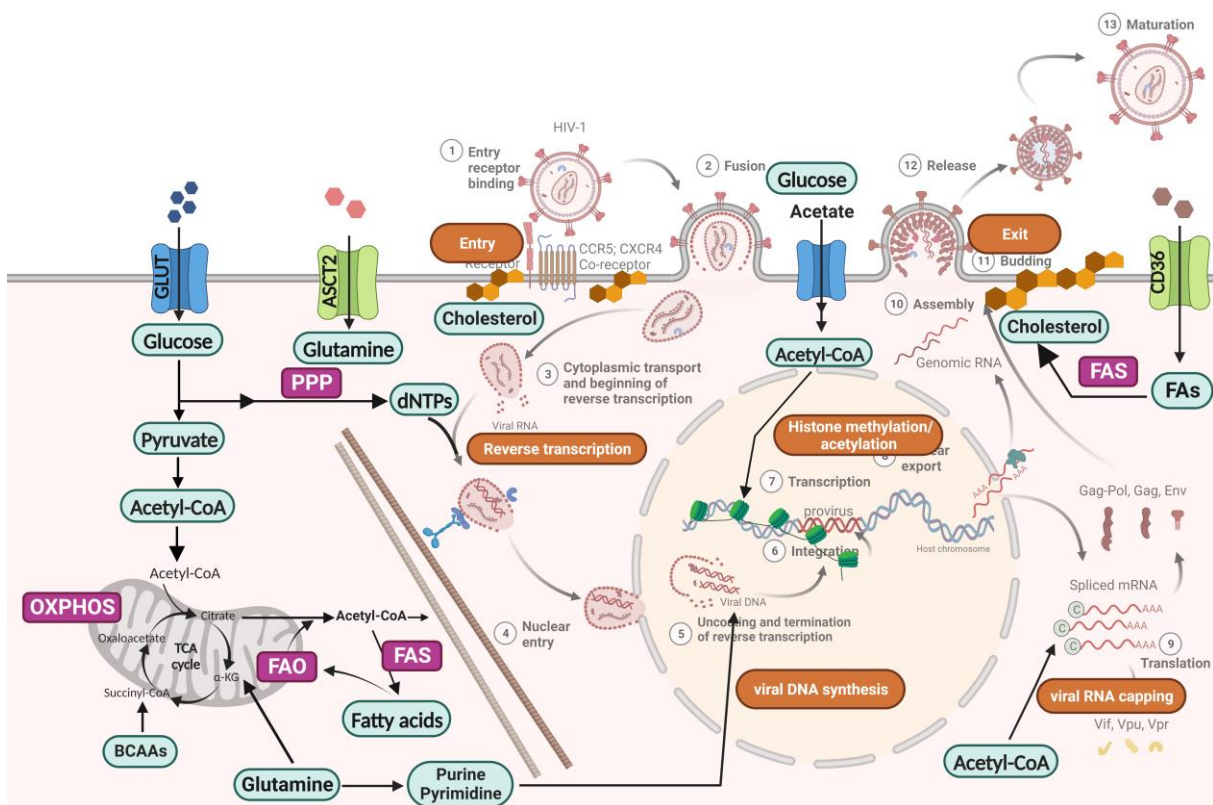
Infection of primary CD4+ T cells seems to rely on glycolysis and glutaminolysis. Treatment with 2-DG, a competitive inhibitor of glucose known to limit cancer cell proliferation and tumor growth, suppressed HIV replication and caused infected cells' death. The association between susceptibility to HIV infection and glycolysis activity has been noted in different independent studies<sup>363,375</sup>. The impact of aerobic vs. oxidative glycolysis on HIV infection remains unknown but UK5099, which inhibits pyruvate transport to the mitochondria, prevented HIV infection of CD4+ T cells, indicating that glucose oxidation may be important for HIV infection. Clerc *et al.* found that HIV infection of CD4+ T cells was more negatively impacted by glutamine deprivation than by glucose. They show that glutaminolysis was the main pathway sustaining OXPHOS in activated T<sub>N</sub> and T<sub>CM</sub><sup>364</sup>.

Additionally, they discovered that infection increased when glucose was redirected into the TCA cycle under inhibited glycolysis. This study demonstrates that a metabolic microenvironment that promotes mitochondrial activity, PPP consumption, a greater NADH/NAD<sup>+</sup> ratio and an OXPHOS biased OXPHOS/glycolysis balance is favorable for HIV infection. Although HIV selectively infects cells with elevated metabolic activity, HIV also induces changes in the metabolism of infected cells. The changes in metabolic pathways are very likely advantageous for viral replication as there are several metabolites that are known to be involved in the retroviral replication cycle. dNTPs are an excellent example. Because the efficiency of HIV reverse transcriptase is dependent on the amount of the intracellular dNTP pool, dNTP metabolism is unquestionably an important regulator of HIV infection (**Figure 11**)<sup>376</sup>. Moreover, by diminishing the dNTP pool, SAMHD1 has been demonstrated to inhibit HIV replication in resting CD4+ T cells and myeloid cells<sup>238</sup>.

Besides glucose, glutamine, fatty acids, acetate, and lactate represent alternate sources of acetyl-CoA. In addition to its important role in energy production, acetyl-CoA abundance leads to acetylation/stabilization of microtubules, which facilitates cytoplasmic transport of viral

products and promote entry and egress from the nucleus in an infected cell<sup>365</sup>. Acetyl-CoA is also a metabolite used by histone acetyltransferases (HATs) to transfer an acetyl group onto a histone lysine. Acetyl-CoA therefore contributes to histone acetylation and the metabolic processes that result in its generation can modulate HIV transcription<sup>377</sup>.

Additionally, acetyl-CoA is a key precursor of *de novo* cholesterol synthesis and cholesterol has been found to aid HIV infection by forming lipid rafts, which are essential for viral entry, trafficking of HIV proteins and viral particles budding<sup>378</sup>. Moreover, cholesterol constitutes up to 40% of the total lipids in the HIV viral membrane and is necessary for infectivity<sup>379–382</sup>. Acetyl-CoA also contributes to FAS and production of myristic acid. This latter binds HIV Gag precursor, a step that is required for Gag to attach to membrane rafts, the assembly of the viral capsid, and the release and maturation of viral particles<sup>383</sup>. Mitochondrial transport blockade of the glycolysis product pyruvate for the production of acetyl-CoA blocks HIV replication<sup>361</sup>.



**Figure 11. Different stages of the HIV-1 replication cycle rely on products of the cellular metabolic pathways.**

Glucose, which is transported into cells through GLUT1, feeds the pentose phosphate pathway (PPP), which participates in the synthesis of dNTPs that are necessary for HIV-1 reverse transcription. Glucose, together with glutamine, also feeds the TCA cycle and OXPHOS in the mitochondria, activities of which are required for early HIV-1 replication. Citrate from the TCA cycle is converted through fatty acid synthesis (FAS) into different intermediates, which are important for the post-translational modification of histones that control HIV-1 transcription and latency, and into fatty acids (such as myristic acid) and cholesterol, which are involved in the formation of membrane domains that are sites of HIV-1 entry and budding. Acetyl-CoA promotes microtubule stabilization for the transport of the viral particle to the nucleus. Acetyl-CoA also participates in histone acetylation and genome transcription. Acetyl-CoA plays an important role in RNA capping, an important step for RNA translation. GLUT1, glucose transporter 1;  $\alpha$ -KG,  $\alpha$ -ketoglutarate; ASCT2, neutral amino acid transporter type 2.

Exogenous addition of  $\alpha$ -KG, another key intermediate of the TCA cycle that can be produced from glucose and glutamine carbons, rescues the decrease in reverse transcription activity observed in glutamine-deprived conditions<sup>364</sup>. Even if the exact metabolic products of glucose and glutamine metabolism necessary for HIV infection are unknown, they are likely important for HIV infection, owing to their contributions to the TCA cycle and the production of biosynthetic intermediates.

## **HIV infection reprograms cellular metabolism**

A growing body of research shows that HIV infection increases aerobic glycolysis in CD4+ T cells and several *in vitro* studies show that various HIV proteins, or viral replication itself, further cause changes in the cell's metabolism. Sorbara *et al.* reported higher expression of GLUT3 and GLUT1 RNA and protein in infected cells, which was coupled with an increase in glucose transport<sup>384</sup>. These observations were further confirmed by Palmer *et al.* by the analysis of HIV-infected PBMC samples from PLWH. These findings also show that glycolysis makers (GLUT1 expression, glucose uptake, intracellular G6P, and L-lactate) remain up-regulated in CD4+ T cells from PLWH, under ART<sup>385</sup>. Gao *et al.* found that activation of CCR5 enhances GLUT1 expression and glycolysis in breast cancer<sup>386</sup>. Whether GP120 interaction with CCR5 may induce similar effects remains unknown, but the binding of gp120 to CD4 and CCR5 or CXCR4 has been demonstrated to cause the release of ATP, which then activates purigenic receptors, an activation required for HIV early replication stages<sup>387</sup>.

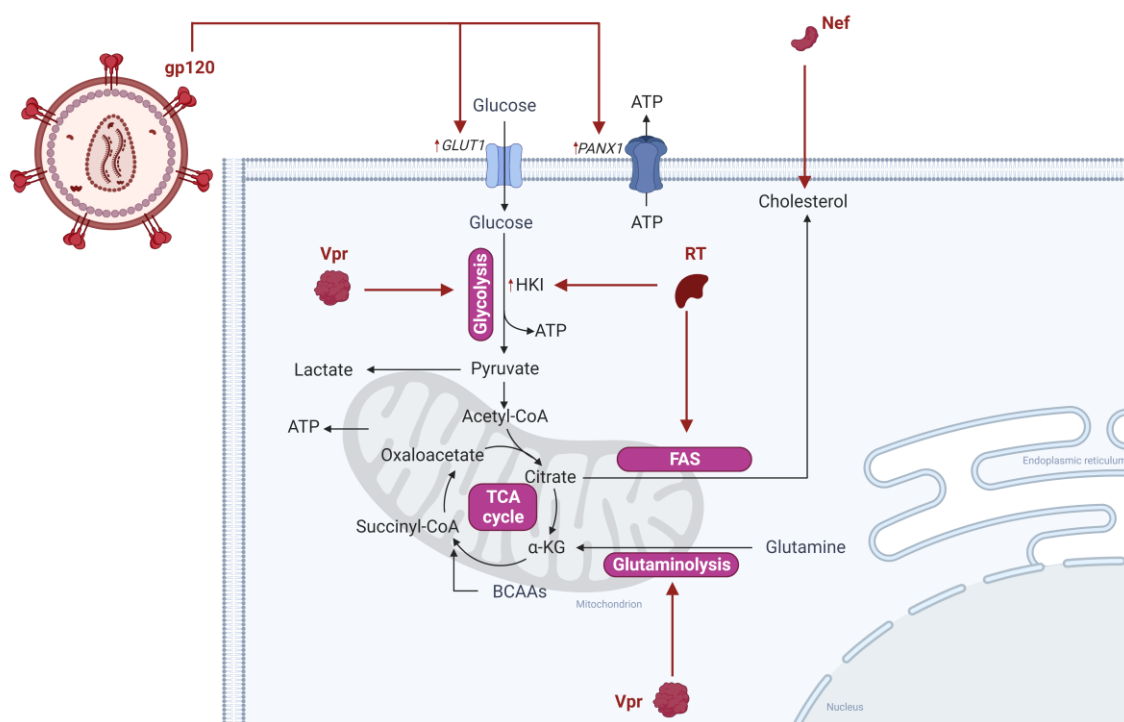
The metabolic reprogramming process is further aided by increased HK activity in HIV-infected CD4+ T cells, and it has been demonstrated that this remodeling depends on viral reverse transcription rather than viral proteins<sup>388</sup>. HIV Vpu alters amino acid metabolism by lowering the surface levels of sodium-coupled neutral amino acid transporter 1 (SNAT1; also known as SLC38A1)<sup>389</sup>. Vpr has also been demonstrated *in vitro* to boost glutamate synthesis by upregulating glycolysis and the TCA cycle<sup>390</sup>. Nef can promote cholesterol production and transport to viral budding sites (**Figure 12**)<sup>391</sup>. Finally, numerous reports show that PLWH on ART, short-term exposure to ARV (PrEP) and PLWH who were never treated with ARV present a decrease in mitochondrial DNA and mitochondrial dysfunction<sup>392–397</sup>. Castellano *et al.* show that HIV infection of macrophages *in vivo* leads in the survival of a limited population of infected cells with metabolic disturbances such as mitochondrial fusion, lipid buildup, and decreased mitochondrial ATP output<sup>398</sup>. However, this impairment has been attributed to non-specific effects of the virus and ART. However, one may argue that the virus modifies mitochondrial activity because it provides specific benefits to the infection, such as promoting the survival of latently infected cells, allowing them to become viral reservoirs. Overall, there are several papers explaining how viruses exploit various methods to alter cell metabolism in order to facilitate viral replication and recent evidence suggest that HIV is not an exception<sup>271,272</sup>.

## **Opportunities for therapeutic intervention targeting cellular metabolism in HIV infection**

As previously noted, there is accumulating evidence for cellular metabolic dysfunction in HIV infection. Notably, immune cells show defects in their metabolic activity that, following the concept of immunometabolism, are very likely associated with their dysfunction. For example,

loss of mitochondrial potential in CD4+ T cells as a result of HIV infection is linked to cell death and CD4+ T cell depletion, and decreased mitochondrial fitness is linked to dysfunctional T<sub>REG</sub> cells found in PLWH who failed to replenish the CD4+ T cell pool after ART. Additionally, GLUT1 is shown to be increased on CD4+ T cells in HIV infection and is linked to systemic immune activation and disease progression, independent of virological suppression with ART<sup>385</sup>.

Work from our team that compared the immunometabolic profiles of HIV-specific CD8+ T cells between HIC and ART-individuals, showed a greater metabolic plasticity of CD8+ T cells from HIC<sup>399</sup>. CD8+ T cells from HICs exhibited greater levels of fatty acid absorption and depended on both glucose metabolism and mitochondrial activity to respond efficiently to HIV antigens, whereas T cells from ART-individuals relied only on glucose metabolism and had a limited capacity to control infection. The link between metabolism profile and HIV control is further supported by a longitudinal analysis of transient HICs, which revealed deregulation of metabolites primarily involved in glycolysis, TCA, and AA metabolism associated with decline in the efficiency of HIV-specific CD8+ T cell responses prior to viral control loss<sup>400</sup>.



**Figure 12. HIV infection reprograms cellular metabolism.**

*Interaction of HIV-1 gp120 with CCR5 or CXCR4 (chemokine receptor 4) activates the pannexin 1 (PANX1) channel, which results in increased extracellular levels of ATP that binds to purinergic receptors and facilitates viral entry. Surface expression levels of GLUT1 might also be increased by interaction of gp120 with CCR5, which results in increased intracellular glucose concentrations. Reverse transcription of HIV-1 may promote glycolysis by increasing the levels of hexokinase 1 (HK1). HIV-1 Vpr promotes the consumption of glucose and glutamine in the TCA cycle. HIV-1 replication and Nef have been suggested to promote FAS and the synthesis of cholesterol (adapted from Sáez-Cirión A, Sereti I. Nat Rev Immunol. 2021<sup>747</sup>).*

HIC CD8+ T lymphocytes were shown to activate closely related metabolic pathways centered on eIF2, mTOR, and PI3K<sup>401</sup>. Transcripts associated with oxidative stress and hypoxia were also upregulated in HIC cells in this study. Along those lines, our team's study revealed that HICs had lower levels of HIF-1A gene expression and higher levels of Von Hippel-Lindau tumor-suppressor protein (VHL), a HIF-1 $\alpha$  counteracting gene, than ART-patients, demonstrating that HIC cells are less primed for glycolysis and activation<sup>399</sup>. Accordingly, Trautmann *et al.* have demonstrated that the susceptibility of HIV-specific CD8+ T cells to apoptosis in early HIV infection was associated with a metabolic state in which these cells exhaust their mitochondria to sustain their proliferation<sup>402</sup>.

Recent research has linked this NK cell dysfunction to impaired cellular metabolism<sup>403,404</sup>. Lipid accumulation in NK cells led to altered gene expression, the downregulation of MYC and mTORC1 signaling and decreased rates of glycolysis and OXPHOS<sup>405</sup>. NK cells with lipid accumulation fail to kill tumor cells in part because they fail to correctly form a synapse with target cells; the cytotoxic machinery does not traffic to the NK cell-tumor cell synapse<sup>405</sup>. NK cells reorganize their mitochondria for immune synapse formation for rapid energy use, thus defective mitochondria result in NK cell dysfunction<sup>406,407</sup>. Canonical NK cells and adaptive CD57+NKG2C+ NK cells, which increase in frequency after persistent HIV infection, show mitochondrial inefficiency and decreased OXPHOS, hampering their capacity to produce IFN- $\gamma$ <sup>408</sup>. IL-15 pretreatment of NK cells was able to improve their metabolic activities and restore their function, similar to what was seen for IL-15-treated CD8+ T cells.

The role of metabolism and metabolites in infectious diseases and immune response, although relatively recent, is being under an extensive exploration in various research and clinical specialties. Moreover, cellular metabolism is thought to be a solution for various diseases that can be improved by boosting the immune system (i.e. cancer, chronic infections) or by dampening specific functions of the immune system (i.e. autoimmunity, diabetes). The essential role of glucose metabolism, driven by Glut1, mTOR, and HIF-1 $\alpha$ , in HIV replication, latency, and inflammatory responses are being better understood, inspiring novel treatment options. Therefore, more data on the multiple metabolic pathways, such as glucose, lipid, and AA metabolism could open possibilities of targeting cellular metabolism as a novel therapeutic strategy for a cure. A promising observation is the finding that metabolic reprogramming through increased fatty acid uptake and mitochondrial respiratory capacity together, via mitochondrial antioxidant treatment and IL-15 treatment, is associated with HIV-suppressing capacity<sup>399,409</sup>.

Lastly, more comprehensive observations regarding metabolic profiles in PLWH with varying clinical characteristics, such as those with optimal immune recovery or not, HICs and PTCs, could be a valuable asset that could enable the use of cellular metabolism as a treatment target toward improved patient outcomes.

# Cytokines

## Cytokines in the immune system

Cytokines are secreted glycoproteins of the immune system that act as a category of signaling molecules that mediate and regulate immunity, inflammation and hematopoiesis of multiple cell lineages of both innate and adaptive immune systems<sup>410</sup>. Cytokines are produced throughout the body by cells of diverse embryological origin. Cytokine is a general name; other names are defined based on their presumed function, cell of secretion, or target of action. Cytokines include lymphokines (cytokines secreted by lymphocytes), monokines (cytokines secreted by monocytes and macrophages), interleukins (cytokines secreted by one leukocyte and acting on other leukocytes) and chemokines (cytokines with chemotactic activities).

A cytokine may have an i) autocrine action by binding to receptors on the membrane of the same cell that produced it, ii) paracrine action by binding to receptors on a target cell in proximity to the producer cell, or iii) endocrine action by traveling through bloodstream and acting on target cells in distant parts of the body. Because of the following characteristics, cytokines can influence cellular activity in a coordinated interactive manner : cytokines are i) pleiotropic, which means that one cytokine can mediate multiple functions; ii) redundant, which occurs when different cytokines can mediate the same or similar functions; iii) synergistic, which occurs when the combined effect of two cytokines on cellular activity is greater than the additive effects of individual cytokines; but also iv) antagonists, which occurs when the effects of one cytokine inhibit or offset the effects of another cytokine. A highly complex network of cytokines operates to regulate the immune system. This network can therefore be redundant, pleiotropic, synergetic and/or antagonist, and can operate in an autocrine, paracrine and/or endocrine manner to stimulate or suppress cellular proliferation and differentiation and to modulate immune function.

Type I cytokines comprise numerous interleukins as well as several growth and hematopoietic factors. They all share one similarity in their basic structure; they have four antiparallel  $\alpha$  helices, with two long and one short loop connections arranged in an up–up/down–down configuration. Because of this structure, these cytokines have also been referred to as the  $\alpha$ -helical bundle cytokine family. This type of cytokines utilize receptors of the cytokine receptor family class I. The extracellular moieties of these receptors contain one or more copies of a domain with a conserved disulfide bridge and a membrane-proximal sequence of tryptophan-serine-X-tryptophan-serine (WSXWS). The  $\gamma$ -chain ( $\gamma$ c) family (also known as the interleukin-2 (IL-2) family) which includes IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, is an important family of type I cytokines. Type I cytokines, in general, promote the formation of a robust cellular immune response, whereas type II cytokines promote the development of a strong humoral immunological response. Some of these type I and type II cytokines regulate each other. IFN- $\gamma$  and IL-12 (type I cytokines), for example, lower the levels of type II cytokines whereas IL-4 and IL-10 (type II cytokines) lower the levels of type I cytokines<sup>411</sup>.

The  $\gamma$ c family is named based on the common usage of a shared cell receptor component,  $\gamma$ c (also known as IL-2R $\gamma$  and CD132). For example, the IL-2 receptor (IL-2R) is composed of three chains (IL-2R $\alpha$ , IL-2R $\beta$ , and IL-2R $\gamma$ ) that when combined form the high-affinity IL-2R. The sharing of  $\gamma$ c across the receptors for members of this family may be a mechanism for generating overlapping activities, but it may also be the basis for this family of cytokines'

potential to compete for the recruitment of  $\gamma_c$ .  $\gamma_c$  is constitutively expressed by all lymphoid cells<sup>412</sup>. This group of cytokines has a wide range of pleiotropic effects on the immune system, encompassing both the innate and adaptive immune systems. These cytokines jointly contribute to the formation of T, B, NK cells and ILCs, promote either cell survival or cell death of immune populations depending on the context, and critically modulating cellular population differentiation into more terminally differentiated cells.

The  $\gamma_c$  cytokines IL-2, IL-7, IL-15 and IL-21 play a key role in the support of T cell proliferation, activation, survival and function during immune responses and homeostatic expansion. Consequently, these cytokines are administered *in vivo* to certain persons with lymphopenia, immune dysfunction, in combination to ART, or in combination with vaccinations to enhance homeostatic peripheral expansion, improve T cell counts and function<sup>413–420</sup>.

### **Mechanisms of cytokine signaling: the JAK-STAT signaling pathway**

The cloning of cytokine receptor genes in 1990 led to better understanding of cytokines tropism and pleiotropic effects<sup>421</sup>. What was less obvious was how cytokine receptor activation resulted in membrane-to-cytoplasmic communication and the fast increase of gene expression. The finding of the crucial involvement of a novel family of tyrosine kinases, Janus kinases (JAKs), for IFN signaling resulted from investigations employing a series of mutagenized cell lines that failed to signal through type I and type II IFNs<sup>422</sup>. Other investigations permitted the identification of signal transducer and activator of transcription proteins and their involvement in IFN signaling, establishing the JAK-STAT paradigm<sup>423</sup>.

The JAK/STAT pathway is a signaling cascade that contributes to the regulation of differentiation, proliferation, migration, survival or cytotoxicity in response to more than 50 cytokines, growth factors and hormones<sup>424–426</sup>. This signaling pathway requires only three elements (receptor, kinase and transcription factor) to elicit a response (**Figure 13**). The JAK/STAT signaling cascade transports extracellular signals from the cell membrane to the nucleus via various steps. In the canonical signaling cascade, signaling of  $\gamma_c$  cytokines is triggered by hetero-dimerization of a proprietary cytokine receptor with the shared  $\gamma_c$  chain. Cytokine binding causes receptor subunit aggregation, which brings JAKs together and allows them to phosphorylate and activate each other. All specific receptors of the  $\gamma_c$  cytokine family, i.e. IL-2R $\beta$ , IL-4R $\alpha$ , IL-7R $\alpha$ , IL-9R $\alpha$ , and IL-21R, are associated with the protein tyrosine kinase JAK1. The  $\gamma_c$  receptor, on the other hand, is constitutively associated with JAK3. IL-2R $\alpha$  and IL-15R $\alpha$  are the exceptions as they are not associated with any kinases and only serve to increase the affinity of the receptor complex for cytokine binding. Activation of the JAK tyrosine kinases is the first and critical step occurring upon receptor engagement. After stimulation, JAKs phosphorylate receptor subunits on tyrosine residues, allowing proteins with SRC homology-2 (SH2) or phosphotyrosine-binding (PTB) domains to be recruited. JAKs can also phosphorylate these proteins. Phosphorylation causes the activation of several metabolic processes. Importantly, cytokine receptor phosphorylation creates docking sites for STAT molecules, a type of SH2-containing transcription factors. The juxtaposition of JAKs and STATs, according to the canonical pathway of signaling, allows STAT dimers to dissociate from the membrane complex and migrate to the nucleus, where they bind specific DNA-motifs modulating the expression of genes, and participate in driving the expression of cytokine responsive genes.

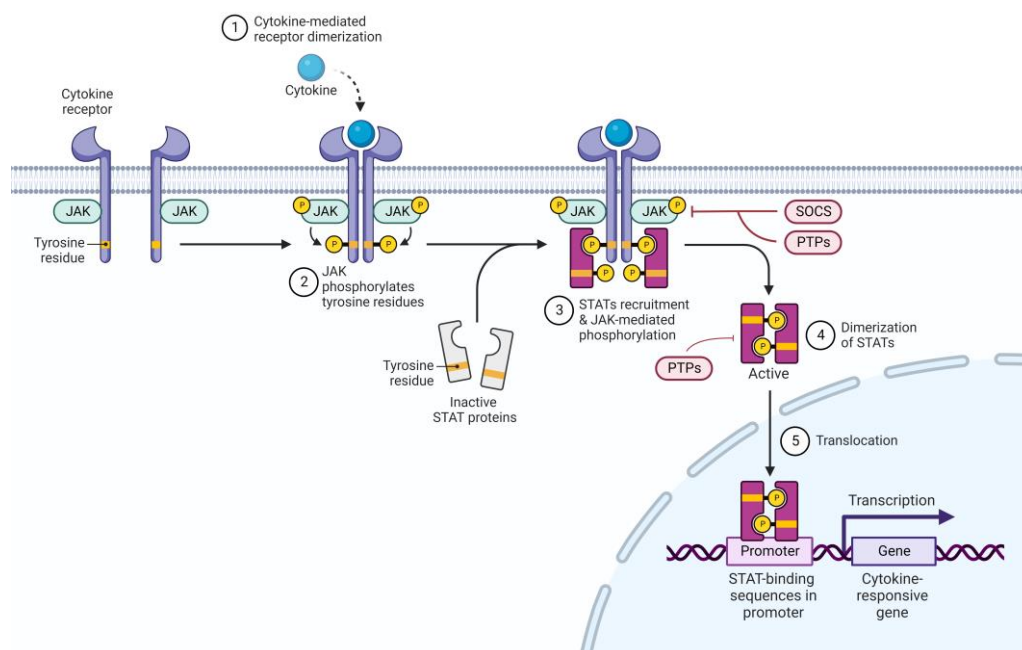


Several non-canonical pathways have also been reported; such as kinase-independent activities of JAKs, the formation of higher order STAT tetramers or multifactorial complexes with other transcription factors, and pathways based on un-phosphorylated STAT proteins (U-STATs)<sup>427</sup>. Additionally, STATs can cause chromatin remodeling by promoting the deposition of permissive or repressive epigenetic alterations<sup>425</sup>.

The vast genomic rearrangement and selective mutation that resulted in mammals having four genes for JAK homologs (JAK1, JAK2, JAK3, and TYK2) and seven for STATs (STAT1-4, STAT5A, STAT5B, and STAT6) in their genomes eventually explain some of the pleiotropic effects associated with this system. STATs proteins are amongst the most conserved and potent transcription factors. JAK3 was the last member of the JAK kinase family to be identified, and its expression is mostly limited to lymphoid and myeloid cells. Depending on the cytokine or growth factor signal, different combinations of JAKs and STATs are activated with a high degree of specificity. JAK/STAT proteins are ubiquitously expressed and are involved in the regulation and maintenance of a range of fundamental biological processes including apoptosis, proliferation, the immune response, and inflammation. Beyond activation of JAKs and STATs,  $\gamma$  family cytokines activate other signaling pathways such as MAPK and PI3K cascades.

JAKs and STATs may be inactivated by suppressor of cytokine signaling (SOCS) proteins. These proteins, whose expression is induced by STATs, act as a negative-feedback loop to switch off the pathway. Other important negative regulators of JAK/STAT signaling are protein tyrosine phosphatases (PTPs), which also inactivate JAKs and STATs.

I will described next more in detail the role of the cytokines that I have studied during my PhD work.



**Figure 13. JAK/STAT signaling. Activation of JAK/STAT signaling pathways.**

(1) Cytokines and growth factors bind to their corresponding receptors, triggering receptor dimerization and recruitment of related JAKs. (2) JAK activation leads to tyrosine phosphorylation of the receptors and the creation of docking sites for STATs. (3) STATs are phosphorylated by tyrosine kinases. (4) STATs disengage from the receptor to form homodimers or heterodimers, (5) which translocate to the nucleus, bind to DNA, and modulate gene transcription.

### **Role of $\gamma$ c cytokines in T cell regulation**

The  $\gamma$ c cytokine family plays unique and non-redundant roles in the formation and differentiation of T lymphocytes as well as other aspects of the adaptive immune system. NK and T cells deficiencies associated with  $\gamma$ c gene (*IL2RG*) mutations and with X-linked severe combined immunodeficiency (XSCID) illustrate the importance of  $\gamma$ c cytokines for the development of these cells in humans<sup>428</sup>.

### **Interleukin-2**

IL-2 is a pro-inflammatory cytokine that is produced by CD4<sup>+</sup> T cells, especially T<sub>H1</sub> cells, and in lesser amounts by CD8<sup>+</sup> T cells, B cells, activated DCs, natural killer T (NKT) and mast cells<sup>429</sup>. IL-2 expression is regulated at the transcriptional and posttranscriptional levels and depends on upregulation of NF-AT, NF- $\kappa$ B, Activator Protein 1 (AP-1) among other transcriptional activators<sup>430,431</sup>. The expression of the transcriptional repressor B lymphocyte-induced maturation protein-1 (Blimp-1) and the T-box transcription factor (T-bet; T-box expressed in T cells) blocks IL-2 transcription<sup>432,433</sup>.

Three classes of receptors bind IL-2 with low, intermediate, or high affinity (**Figure 14**). IL-2R $\alpha$  (also known as CD25) alone constitutes a low-affinity receptor; the combination of IL-2R $\beta$  (also known as CD122) and IL-2R $\gamma$  ( $\gamma$ c or CD132) forms intermediate-affinity receptors; all three chains combine to form a high-affinity receptor<sup>434</sup>. The expression of the three IL-2R chains is independently regulated. With the exception of T<sub>REG</sub> cells, which constitutively express IL-2R $\alpha$ , which is consistent with the persistent autoantigen TCR stimulation and IL-2-dependent signals in T<sub>REG</sub> cells, this receptor chain is absent or minimally expressed on resting T cells but is induced following TCR engagement, cytokines (such as IL-15), several viral proteins as well as a range of other stimuli<sup>431,435,436</sup>. Additionally, NK and activated B cells were also shown to express IL-2R $\alpha$ . Finally, IL-2R $\alpha$  is expressed during T and B cell development in the thymus and bone marrow, respectively, but also by CD4-CD8- thymocytes, as well as B220<sup>+</sup> progenitor B cells during ontogeny. The involvement of IL-2R $\alpha$  in these processes, however, remains not fully understood. The existence of a soluble IL-2R $\alpha$  has also been described<sup>437</sup>.

IL-2R $\beta$  is constitutively expressed by at least some resting T cells, B and NK cells, monocytes, dendritic epidermal T cells (DETCs) and neutrophils<sup>438-441</sup>. For T cells, stimulation with a number of different stimuli can greatly induce IL-2R $\beta$  expression<sup>438</sup>. These positive feedback loops guarantee that the high-affinity IL-2 receptor complexes are only formed in activated T cells, which further increase their responsiveness to IL-2<sup>429</sup>.

IL-2 primarily signals in *cis*, but can also be presented in *trans*, where IL-2 bound to IL-2R $\alpha$  on one cell stimulates another cell that expresses IL-2R $\beta$  and  $\gamma$ c. However, IL-2 binds with relatively low affinity to IL-2R $\alpha$  with rapid on and off rates therefore *cis*-based IL-2 signaling is likely favored whenever a cell expresses all three chains whereas *trans*-signaling presumably necessitates rather high concentrations of IL-2<sup>442</sup>. During an immune response, IL-2 is consumed in an autocrine or paracrine manner by cells in close proximity bearing IL-2R $\alpha$ . Although typically considered a T cell surface marker, IL-2R $\alpha$  (CD25) is also found in activated monocytes. However, it is unclear whether the expression of IL-2R $\alpha$  in these cells reflects their ability to *trans*-present IL-2 or than responsiveness to IL-2.

The formation of the high-affinity IL-2-IL-2R complex results in signal transduction via the tyrosine kinases JAK1 and JAK3, which are associated with IL-2R $\beta$  and  $\gamma$ c, respectively<sup>443</sup>.

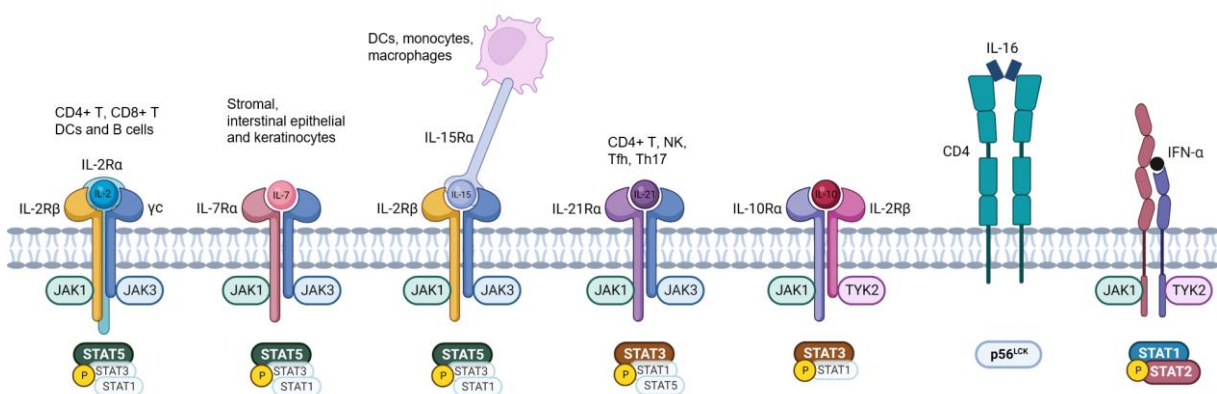
Phosphorylation specific residues in IL-2R $\beta$  allows association of the Shc adaptor protein which provides a platform for Ras-MAP kinase activation and Src signaling<sup>444</sup>.

The phosphorylation of adjacent tyrosine residues causes the recruitment of STAT1, STAT3, STAT5A, and STAT5B, with STAT5 proteins exhibiting the most potent and sustained activation<sup>444,445</sup>. After docking on IL-2R $\beta$ , phosphorylated STAT5A and STAT5B translocate to the nucleus and bind to target genes essential for effector cell function, T cell growth as well as differentiation<sup>444-446</sup>. Additionally, IL-2 stimulates the PI3K/PTEN/Akt/mTOR signaling pathway, which supports cell growth and survival<sup>447-450</sup>.

IL-2 plays an essential role in controlling immune homeostasis. In the onset of the immune response, IL-2 is the third essential signal, in addition to TCR triggering plus positive co-stimulation, that supports the expansion of peripheral immune cells and promotes the survival of effector cells. IL-2 was detected as a growth factor for T cells, but has subsequently been shown to help the growth of many hematopoietic lines. The biological actions of IL-2 appear to be highly tuned to generate cell type-specific responses, which is a distinguishing feature of this cytokine.

IL-2 is typically thought of as an activator since it induces more genes than the ones it represses. A miRNA, miR-182, that is induced by IL-2 is an inhibitor of the transcription factor FOXO1, which blocks cell-cycle progression in resting T cells, thereby helping to mediate the clonal expansion of activated helper T cells<sup>451</sup>. IL-2 plays an important role in T<sub>REG</sub> differentiation and homeostasis, whilst in effector T cells, IL-2 increases differentiation, proliferation, and cell growth<sup>452</sup>. Although other  $\gamma$ c cytokines such as IL-4, IL-7, IL-15 and IL-21 can also promote the survival of T<sub>REG</sub> cells and rescue them from apoptosis *in vitro*, only IL-2 induces their proliferation and clonal expansion.

Contrary to what is seen for effector T cells, IL-2 does not induce rapid proliferation in T<sub>REG</sub> cells, emphasizing the exclusivity of its actions based on the subset of cells it acts<sup>453</sup>. Given the very distinct phenotypes of T<sub>REG</sub> cells and effector T cells, and since IL-2 signaling pathways like mTOR have opposing effects on the development of these cells, it is unlikely that IL-2 activates and supports similar pathways in T<sub>REG</sub> and effector cells. Therefore, it has been suggested that IL-2 induces different signals in different T cell subsets.



**Figure 14. Cytokine receptors.**

The receptors for IL-2, IL-7, IL-15, IL-21, IL-10, IL-16, and IFN- $\alpha$  are shown. Only two of these cytokines have three receptor chains: IL-2 and IL-15. These two cytokine receptors share the same cytokine receptor  $\gamma$ -chain and IL-2R $\beta$ . IL-10, IL-16, and IFN- $\alpha$  are the only cytokines that do not signal through a receptor that contains  $\gamma$ c. There are three types of IL-2 receptors that bind IL-2 with varying degrees of affinity: low affinity (IL-2Ra alone), intermediate affinity (IL-2R $\beta$  and  $\gamma$ c), and high affinity (IL-2Ra, IL-2R $\beta$  and  $\gamma$ c). Each  $\gamma$ c family cytokine receptor activates Janus kinase 1 (JAK1) and JAK3. The key STAT proteins activated by these cytokine receptors are highlighted in colors.

IL-2 type of signaling depends on quantifiable factors such as the concentration and the duration for which cells are exposed to this cytokines<sup>454</sup>. Prolonged IL-2 signaling favors the generation of effector CD8+ T cells and results in a reduction in number of memory CD8+ T cells<sup>455</sup>. IL-2 effects also depend on its mode of action, meaning whether it acts in a paracrine or autocrine way. For example, autocrine IL-2 secretion by CD8+ T cells favors the generation of memory CD8+ T cells but paracrine IL-2 derived from DCs or CD4+ T cells gives rise to terminally differentiated effector cells which express effector molecules such as perforin, granzyme B, IFN- $\gamma$ , and low amounts of IL-2<sup>456,457</sup>. In NK cells, IL-2 induction of IL-2R $\beta$  not only further increases responsiveness to IL-2 but also to IL-15 (due to a shared receptor chain, see below), thus promoting NK activity. Finally, IL-2 was also found to stimulate B cell immunoglobulin synthesis<sup>431</sup>.

Overall, IL-2 deficiency is associated with a decrease in the number and function of T<sub>REG</sub> cells and is consequently linked to autoimmunity. The ability of T<sub>REG</sub> cells to successfully compete with effector CD4+ T cells for the available IL-2, owing to the high IL-2R $\alpha$  expression, is one reason for their suppressive actions<sup>458</sup>. Moreover, IL-2 is a key factor in defining a specific effector T cell subset fate<sup>459</sup>. IL-2 regulates the fate of CD4+ T cells by modulating the expression of important cytokine receptors, transcription factors, chromatin regulators, and effector cytokines. For instance, IL-2 plays an important role in promoting the differentiation of T<sub>H1</sub> versus T<sub>H2</sub> cells through the overexpression of T-bet<sup>460</sup>. However, IL-2 is a repressor of T<sub>FH</sub>, based on its repression of Bcl-6 expression, and of T<sub>H17</sub> differentiation<sup>461,462</sup>.

IL-2 can also induce T cell terminal differentiation and exhaustion<sup>463</sup>. Although IL-2 is considered to be a survival and growth-promoting cytokine, it also mediates activation-induced cell death (AICD) late in the immune response, depending on the cellular and activation context, which constitutes for its role in controlling self-reactive T cells and T cell responses<sup>464</sup>. It is important to note that in the first phase of the activation of T lymphocytes, the lack of IL-2 will also result in apoptosis. Indeed, increased expression of pro-apoptotic protein Bim and a decreased phosphorylation of the pro-apoptotic protein Bad due to the lack of IL-2 and lower Akt activity, results in apoptosis of effector cells<sup>465</sup>.

Overall, production of adequate levels of IL-2 is critical for the proper function of CD4+ T lymphocytes, which, in turn and with the direct action of IL-2, regulate the responses of other lymphocyte populations such as CD8+ T, NK and B cells. Other factors such as the target cell type, the location, the binding affinity, and the co-secretion of other soluble mediators will influence IL-2 mediated response.

### **Interleukin-7**

IL-7 is a cytokine that was initially identified as a new growth factor responsible for increasing the proliferation of murine B cell precursors in a long-term bone marrow culture system, but has since been revealed to be crucial for lymphocyte development and survival<sup>466,467</sup>. IL-7 is thought to be the first cytokine immature thymocytes encounter during their development in the thymus. Contrary to the other  $\gamma$ c cytokines, that are mostly produced after immune cell activation, IL-7 is generated constitutively by a wide variety of cells including marrow and thymic stromal and epithelial cells, as well as in kidney, spleen and keratinocytes<sup>468</sup>. This is consistent with its role in the maintenance of function in both immature and mature lymphocytes. It is also produced by stromal and intestinal epithelial cells, keratinocytes, DCs, neurons as well as mononuclear phagocytes, but not by lymphoid cells<sup>469,470</sup>.

IL-7 receptor is formed by the  $\gamma$ c plus the IL-7R $\alpha$  chain (CD127). IL-7R $\alpha$  is expressed at high levels in early thymocytes and most mature T cells excluding effector T cells and senescent memory populations<sup>471</sup>. Additionally, IL-7R $\alpha$  is found on DCs, monocytes and a subset of developing B cells<sup>469</sup>.

IL-7R signals through the JAK1/JAK3/STAT5, PI3K/Akt/mTOR and MAPK/ERK signaling pathways<sup>472</sup>. These pathways are shared with IL-2, despite each cytokine harboring distinct roles in the regulation of immune system development and function, which further emphasizes the complex fine-tuning behind the JAK/STAT signaling pathway as well as the significance of the non-canonical routes that both of these cytokines employ<sup>473</sup>. While the other  $\gamma$ c cytokine receptors increase in expression following TCR stimulation, IL-7R $\alpha$  is expressed by both naïve and memory resting T cells, but its expression is downregulated after cell stimulation, likely to optimize the total number of T cells supported by IL-7<sup>474</sup>. IL-2 as well as other pro-survival cytokines can temporarily decrease IL-7R $\alpha$  availability on T cells<sup>474</sup>. Consequently, it is assumed that IL-7 is not necessary for the function of activated T cells. After activation, naïve CD4+ T cells downregulate IL-7R $\alpha$ , transition to the effector CD4+ T cell stage, and receive survival signals from TCR stimulation along with to other environmental factors including IL-2<sup>475</sup>. However, a portion of memory effector cells re-express CD127 and develop into long-lived memory cells<sup>476,477</sup>. Hence, IL-7R $\alpha$  is a marker for the subset of T cells committed to become memory cells<sup>478</sup>.

IL-7 plays a major role in thymocyte survival and proliferation and is essential for proper T cell homeostasis, regulating both thymic output and peripheral regeneration of T cells as well as protecting peripheral T cells from apoptosis<sup>470,479,480</sup>. Among the members of the  $\gamma$ c family of cytokines, IL-7 is the most important for regulating naïve and memory CD4+ and CD8+ T cells homeostasis<sup>477,481,482</sup>. T cell homeostasis in the peripheral lymphoid compartment is strictly controlled by turnover, survival, and death in the absence of external disruption. The sole basal TCR stimulation however, is insufficient to keep T cells alive. IL-7 employs two mechanisms to promote T cell homeostasis. By triggering the PI3K-Akt signaling pathway, increases the production of survival factors including Bcl-2, and myeloid cell leukemia sequence 1 (Mcl-1), which can significantly inhibit the mitochondrial apoptotic pathway, while suppressing the expression of the pro-apoptotic proteins Bax, Bim, and Bad<sup>468,477,483</sup>. Naïve CD4+ T cells are critically dependent on IL-7 for their homeostasis. It promotes their expansion while maintaining the naïve phenotype<sup>151,484</sup>. As a result, naïve CD4+ T cells display enhanced cell surface expression of IL-7R. *In vivo*, IL-7 is only available in trace amounts in the serum in the absence of infection, which has been suggested to serve a regulatory function by enabling a maximum number of periphery naïve cells to continually compete with one another for IL-7-induced survival signals, with un-signaled T cells condemned to extinct<sup>474,485</sup>. Additionally, since cells downregulate the expression of the IL-7 receptor upon IL-7/IL-7R engagement, excessive quantities of this cytokine can be detrimental for T cell homeostasis<sup>474</sup>.

In addition to its crucial function in regulating T cell development in the thymus and naïve T cell homeostasis in the periphery, IL-7 regulates the formation of memory T cells primarily through increasing T cell survival during acute infections<sup>468,486</sup>. Moreover, IL-7 allows the homeostatic maintenance of memory T cells in those situations lacking of the persistent cognate antigen<sup>477</sup>.

Finally, IL-7 also appears to play a role in the generation of CTL, including the regulation of cytolytic activity of intestinal epithelial lymphocytes<sup>487</sup>. It participates in the T cell response following antigenic stimulation and can promote the development of active lytic CD8+ killer T

lymphocytes as well as the activation of NK cells to become lymphokine activated killer (LAK) cells<sup>488</sup>. IL-7 can also limit the development of T cell exhaustion during chronic antigen stimulation<sup>489</sup>. In this sense, IL-7 decreases the PD-1 expression in chronic viral infection models<sup>490</sup>. Moreover, this  $\gamma$ c cytokine downregulates a repressor of cytokine signaling, Socs3, a members of the SOCS (suppressors of cytokine signaling) family, resulting in amplified cytokine production and increased T cell effector functions<sup>491</sup>. Furthermore, IL-7 was suggested to be involved in multiple stages of the development of B cell progenitor, including its commitment, survival, differentiation, and proliferation<sup>492</sup>. Other roles for IL-7 important for other immune cells function, such as DCs, have been described<sup>493</sup>.

### **Interleukin-15**

The pluripotent cytokine IL-15 was initially identified as a T cell growth factor that not only performed similarly to IL-2 but also used parts of the IL-2 receptor for signaling<sup>494</sup>. IL-15 mRNA is expressed in a wide range of both hematopoietic and non-hematopoietic cells: monocytes, macrophages and DCs, keratinocytes, fibroblasts, myocytes, stromal and nerve cells<sup>495</sup>. However, its protein expression is limited and mainly detected on APCs such as monocytes, macrophages and DCs<sup>496</sup>. The disparity between ubiquitous IL-15 transcript expression and limited protein production is due to several checkpoints during transcription, translation, and intracellular trafficking<sup>497</sup>. On a translational level, in particular, there are multiple triplet repeats of adenosine, uradine, guanosine (AUGs) in the 5' untranslated portion of the IL-15 mRNA that restrict its translation. IL-15 is not typically produced by T cells, however, human T-lymphotropic virus 1 (HTLV-1)-transformed T cells and some T cell lines express IL-15 mRNA and produce abundant IL-15<sup>498</sup>.

Both IL-2 and IL-15 act through receptors that share the same  $\beta$ - and common  $\gamma$ -chain but employ different  $\alpha$ -chains. IL-15 can bind to the IL-15R $\alpha$  (CD215) with high affinity. CD215 retains IL-15 on the cell surface, amplifies the affinity of this cytokine for IL-2R $\beta$  compared with its free form, and *trans*-presents it to neighboring cells that express IL-2R $\beta$  and  $\gamma$ c such as memory CD8+ T cells<sup>499</sup>. The IL-15/IL-15R $\alpha$  complex can also be cleaved off the cell surface, thus allowing IL-15 to be “*trans*-presented” in the absence of a presenting cell. Although IL-15 is capable of forming a low-affinity complex with IL-2R $\beta$  and  $\gamma$ c in the absence of IL-15R $\alpha$ , which is not required for signaling, the binding of IL-15 to IL-15R $\alpha$  increases the affinity of IL-15 for IL-2R $\beta$ / $\gamma$ c by approximately 100 folds over IL-15 alone<sup>500</sup>. IL-15 signals similarly to IL-2 and IL-7 via the JAK1/JAK3/STAT5 signaling pathway, which then recruits the PI3K/Akt/mTOR and MAPK/ERK cascades<sup>429,501</sup>.

Given their shared receptor use, the activities of IL-2 and IL-15 are very comparable, particularly in terms of their effects on lymphoid cells. IL-15 has similar effects to IL-2 in terms of T cell proliferation and survival, but acts preferentially on CD8+ rather than CD4+ T cells<sup>468</sup>. IL-15, like IL-2 but to a greater extent, can enhance the cytolytic activity of NK and CD8+ T cells. Another difference between IL-2 and IL-15 is that whereas IL-2 encourages terminal differentiation and ultimately leads to the elimination of antigen-activated effector cells through AICD, IL-15 inhibits AICD and promotes the development of long-lived memory T cells as well as their maintenance through homeostatic proliferation, i.e. proliferation induced as a consequence of T cell depletion<sup>502</sup>. By increasing the expression of anti-apoptotic molecules like Bcl-2 and Mcl-1, and counteracting the action of pro-apoptotic molecules like Bim, IL-15 preserves T cell, and particularly memory T cell, survival<sup>503</sup>. In the absence of IL-15, memory CD8+ T cells show a reduced cell cycle and impaired Bcl-2 expression.

Accordingly, IL-15 was reported to promote the survival of antigen-experienced MHC II independent CD4<sup>+</sup> T cells<sup>468</sup>. While it has been demonstrated that the homeostatic proliferation and maintenance of CD8<sup>+</sup> memory T cells under physiological conditions require both IL-7 and IL-15 jointly, CD4<sup>+</sup> memory T cells only depend on the function of IL-7 for these same features<sup>504-506</sup>. For CD4<sup>+</sup> memory cells, the less evident involvement of IL-15 correlates with lower expression of IL-2R $\beta$  than on memory CD8<sup>+</sup> cells<sup>507</sup>. An exception is made when IL-7 levels are low, where IL-15 has been shown to play a key role in memory CD4<sup>+</sup> T cell homeostasis particularly in non-lymphopenic conditions<sup>468,504</sup>. In lymphopenic conditions however, when higher levels of IL-7 are accessible *in vivo* due to the lack of competition with naïve T cells, both memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells can still be maintained by IL-7 even in the absence of IL-15<sup>508-510</sup>. Finally, IL-15 not only contributes significantly in the maintenance of memory subsets, but also plays a key role in defining the composition of the memory T cell pools. Because IL-7 and IL-15 signals overlap, it is plausible to predict that cells lacking CD127 (IL-7R $\alpha$ ) will become reliant on IL-15 for survival. Accordingly, treatment with multivalent IL-15/IL-15R $\alpha$  complex can promote appearance of CD127<sup>low</sup> cells during the contraction phase of an immune response<sup>511</sup>. Furthermore, IL-15 deficiency leads to the disappearance of CD127<sup>low</sup> memory cells as well as modifications within the CD127<sup>high</sup> effector memory pool, which expresses minimal granzyme B<sup>512</sup>. Therefore, IL-15 not only maintains memory response but also regulates its composition.

IL-15 also appears to be a key cytokine in the host response to infections. *In vitro* studies have shown a significant effect of IL-15 on the number but also function (with respect to IFN- $\gamma$  production and direct cytotoxicity) of CD8<sup>+</sup> T cells<sup>513</sup>. Similarly, mice studies show that IL-15 enhances the effector function of antigen-specific CD8<sup>+</sup> T cells<sup>514</sup>. Interestingly, IL-15 appears to serve modest roles for antiviral CD8<sup>+</sup> T cells during the expansion phase, and memory CD8<sup>+</sup> T cells formed in IL-15- or IL15R $\alpha$ -deficient mice after infection were phenotypically and functionally similar to those generated in IL-15-sufficient mice<sup>515</sup>. However, the lack of IL-15 signals caused memory CD8<sup>+</sup> T cells to gradually decline in number, confirming the importance of IL-15 for the homeostatic proliferation of memory CD8<sup>+</sup> T cells generated after infection<sup>508,515,516</sup>. These observations were corroborated by the finding that administering IL-15-IL-15R $\alpha$  complexes induced strong proliferation of memory CD8<sup>+</sup> T cells<sup>511</sup>.

Although, on its own, IL-15 is not essential for the humoral immune response and antibody production, several studies demonstrate the benefit of IL-15 as an adjuvant to induce maximal antibody responses<sup>517</sup>. IL-15 has been associated with an increase in IgA B cell generation in mucosal tissues<sup>518</sup>. It is also important to note that besides playing an important role in regulating adaptive immune cells, IL-15 is critical for innate lymphocytes development. IL-15 is involved in the development, the persistence and the activation of NKT and  $\gamma/\delta$ T cells<sup>499</sup>. In addition, IL-15 is the main driver of NK cell development as demonstrated by the lack of functional NK cells in mice that lack either IL-15 or IL-15R $\alpha$ <sup>519,520</sup>. Furthermore, IL-15 enhances cytolytic activities of NK cells and induces their IFN- $\gamma$  production<sup>521</sup>.

Finally, IL-15 was found capable of increasing T<sub>H1</sub> or T<sub>H2</sub> development in response to TCR activation in the presence of other essential cytokines<sup>522</sup>. Similar to IL-2, IL-15 has been reported to constrain T<sub>H17</sub> cell generation via STAT5 signaling and an increase in the number of intestinal T<sub>H17</sub> cells in IL-15<sup>-/-</sup> mice was reported<sup>523,524</sup>. Other studies suggested a role for IL-15 in T<sub>REG</sub> development, however, unlike IL-2, IL-15 is not required for the maintenance of this population<sup>525</sup>.

## **Interleukin-21**

IL-21 is the most recently characterized cytokine of the  $\gamma$ c family. IL-21 is produced by a variety of CD4<sup>+</sup> T cell subsets, mainly by T<sub>H17</sub>, T<sub>FH</sub> and NKT cells<sup>526-528</sup>. In the case of HIV infection, CD8<sup>+</sup> T cells were also shown to produce IL-21<sup>527,529</sup>. Of note, CD8<sup>+</sup> IL-21 producing T cells are found enriched in HIV controllers<sup>527,529,530</sup>. Finally, IL-21 has been shown to be produced by NKT cells as well<sup>531</sup>. IL-21 acts on a wide array of cells, including T cells, B cells, NK cells, DCs, macrophages, mast cells and epithelial cells<sup>529</sup>. IL-21 primed DCs have inhibitory effects on CD4<sup>+</sup> T cells, which suggests that the production of IL-21 by activated CD4<sup>+</sup> T cells initiates a DC-mediated negative feedback loop<sup>532</sup>.

Interleukin-21 receptor (IL-21R) is expressed on lympho-hematopoietic cells and is composed of two chains: IL-21R $\alpha$  +  $\gamma$ c. IL-21-induced signal transduction involves both JAK1 and JAK3 kinases<sup>533</sup>. IL-21 activates STAT1, STAT3, and STAT5, with preferential activation of STAT1 and STAT3<sup>533</sup>.

IL-21 has a wide range of functions. As mentioned above, IL-21 is produced by multiple CD4<sup>+</sup> T cell subsets, prominently T<sub>H17</sub>, T<sub>FH</sub> and NKT cells. The cytokine enhances differentiation of some of these subsets, but other immune cells, including B, CD8<sup>+</sup> T and NK cells also respond to IL-21. IL-21 also promotes T<sub>H2</sub> versus T<sub>H1</sub> and T<sub>H17</sub> type of immunity<sup>534-536</sup>. It also favors the expansion of certain T and B cell subpopulations<sup>537-540</sup>. IL-21 supports the terminal differentiation of B cells to plasma cells, but it also serves as a pro-apoptotic factor for incompletely activated B cells<sup>528</sup>. It also facilitates isotype switch to IgG1, IgG3 and IgA, but suppresses IgE generation<sup>541,542</sup>. In addition, B cells acquire cytotoxicity by induction of granzyme B as a consequence of their stimulation with IL-21 cytokine<sup>543</sup>. IL-21 also works in concert with other  $\gamma$ c cytokines. IL-21 promotes the expansion of CD8<sup>+</sup> T cells after IL-7 or IL-15-induced homeostatic proliferation, and thus contributes to CD8<sup>+</sup> T cell memory formation<sup>419</sup>. IL-21, on the other hand, can block IL-15-induced CD28 down-modulation, which promotes naïve CD8<sup>+</sup> T cells to an effector phenotype. As a result, IL-21 maintains CD8<sup>+</sup> T cell reactivity to CD28 ligands<sup>544</sup>. Furthermore, although *IL21r* KO mice show normal acute antiviral CD8<sup>+</sup> T cell responses, IL-21 is essential in preventing the exhaustion of specific CD8<sup>+</sup> T cells, sustained effector responses, and eventual control of chronic infections<sup>545</sup>. Additionally, although it is unknown whether it is the consequence of IL-21 alone or of IL-21's synergistic activity with constitutively expressed IL-7 or IL-15, overexpression of IL21 is associated with a higher count of CD8<sup>+</sup> T cells<sup>546</sup>. IL-21, with IL-15, promotes the generation of NK in the bone marrow<sup>547</sup>. Moreover, IL-21 induces an acceleration of the maturation steps towards the acquisition of a mature NK cell phenotype, including surface expression of Ig-like killer inhibitory receptors (KIR)<sup>548</sup>. Finally, IL-21 can also enhance cytotoxic activity and IFN- $\gamma$  production by activated murine NK cells, but at the expense of their viability, thus limiting their duration of activation<sup>540</sup>. IL-21, being a double-edge sword, also can block IL-15-induced expansion of resting NK cells and promote their apoptosis<sup>540,549</sup>.

## **Additional cytokines**

In addition to  $\gamma$ c cytokines that play mostly a role in T cell homeostasis, other cytokines with distinct signaling pathways and mode of actions are known to influence T cell dynamics<sup>550-553</sup>. Closely related to type I cytokines are IL-10-related cytokines and IFNs (IFNs), which bind type II receptors and form the larger class II cytokine family. Like the  $\gamma$ c family, members of the IL-10 family share a receptor subunit. These subunits share some similarities with type I



cytokine receptors, however, they lack the intact WSXWS motif. IL-10 is the founding member of a family of cytokines that also includes IL-19, IL-20, IL-22, IL-24, IL-26 and different types of IFNs, i.e. type I IFNs, IFN- $\gamma$  and type III IFNs (IL-28 $\alpha$ , IL-28 $\beta$ , and IL-29)<sup>553-555</sup>. They all share the following characteristics: clustering of their encoding genes, analogous genomic structures (having evolved through gene duplication), similar primary and secondary protein structures, and use of similar receptor complexes<sup>556,557</sup>. One of the central functional themes of IL-10 family cytokines is their role in tissue protection<sup>553,558,559</sup>. These cytokines prevent excessive tissue damage caused by bacterial and viral infections as well as pro-inflammatory responses<sup>553,560</sup>.

### **Interleukin-10**

IL-10, commonly referred to as “T cell growth inhibition factor”, is a pleiotropic cytokine that was first described as a secreted "cytokine synthesis inhibitory factor" and a product of T<sub>H2</sub> cells that inhibited cytokine synthesis in T<sub>H1</sub> cells<sup>561</sup>. This cytokine is an essential anti-inflammatory component that plays important roles as a negative regulator of immune responses to reactive antigens. IL-10 can be produced in response to pro-inflammatory signals by virtually all immune cells, including T cells (mainly cytotoxic CD8+ and T<sub>H2</sub> cells), B cells, macrophages, monocytes, DCs, mast cells, eosinophils, neutrophils and NK cells<sup>553,554</sup>. LPS and TNF- $\alpha$  are inducers of IL-10<sup>562,563</sup>.

IL-10 binds to extracellular domain of IL-10R composed of two chains: IL-10R1 and IL-10R2)<sup>564</sup>. IL-10R1 binds IL-10 with high affinity and is specific to the IL-10 receptor, whereas IL-10R2 is also found in the receptors for IL-22, IL-26, IL-28A, IL-28B, and IL-29<sup>552,553</sup>. While the IL-10R2 chain is ubiquitously expressed, IL-10R1 is found mostly on leukocytes, which makes IL-10 the only cytokine in the IL-10 family that predominantly targets leukocytes. IL-10/IL-10R binding leads to phosphorylation of JAK1 and Tyrosine Kinase-2 (TYK2), which are associated with IL-10R1 and IL-10R2 respectively<sup>565</sup>. This allows for the recruitment of STAT3 and to a lesser extent STAT1, engaging therefore the JAK/STAT signaling pathway. IL-10 was also shown to activate other downstream signaling pathways, including PI3K/Akt cascade, which in turn leads to mTORC1 activation<sup>566,567</sup>.

IL-10 is very important for T cell homeostasis. This regulatory cytokine functions primarily as a feedback inhibitor of excessive T cell responses to microbial antigens. The finding that IL-10-deficient mice, maintained under specific-pathogen free conditions, develop autoimmune diseases emphasizes the importance of IL-10 for homeostasis<sup>568</sup>. IL-10-deficient mice develop enterocolitis as a consequence of an excessive immune response to the gut microbiota, a condition phenotypically similar to chronic inflammatory bowel disease (IBD) in humans, and exaggerated inflammatory responses<sup>569</sup>. Similarly, humans with mutations of the *IL10* and *IL10R* genes suffer from IBD<sup>570</sup>. These studies demonstrated the essential role IL-10 has in maintaining homeostatic T cell tolerance and macrophage activation in response to commensal bacteria in the intestine<sup>571</sup>.

During ongoing immune responses against pathogens, IL-10 is produced as a negative feedback mechanism to properly control and limit inflammatory responses<sup>572</sup>. Depending on the nature of the immune response, IL-10 deficiency may result in an accelerated immune response and hence quicker pathogen clearance; on the other hand, it may result in excessive inflammation, which can cause tissue damage or lethality. IL-10 primarily targets APCs, such as monocytes and macrophages, by blocking the production of pro-inflammatory mediators from these cells<sup>573</sup>. IL-10 also suppresses antigen presentation by decreasing the expression of

MHC II, as well as co-stimulatory and adhesion molecules<sup>574</sup>. Additionally, this cytokine also prevents the maturation of DCs<sup>575</sup>. Furthermore, IL-10 suppresses the synthesis and/or release of cytokines necessary for CD4+ T cell development<sup>576</sup>. Finally, besides indirectly dampening the excessive T<sub>H1</sub>, NK and CD8+ T cells immune responses, IL-10 can also directly reduce cytokine production and proliferation of CD4+ T cells<sup>577</sup>. However, IL-10 promotes the survival and action of T<sub>REG</sub> cells<sup>578</sup>. Surprisingly however, IL-10–stimulatory effects on NK and CD8+ T cells, such as enhanced proliferation, cytotoxic activity, and IFN- $\gamma$  production, have also been described<sup>579–581</sup>. Additionally, recent work by Yogev *et al.* describes a role for IL-10 in promoting inflammation in the central nervous system by sustaining effector T survival<sup>582</sup>. Moreover, in certain circumstances, IL-10-mediated negative feedback promotes persistent infection by dampening the immune response sufficiently to preclude pathogen clearance<sup>572</sup>. This is the case, for example, with the IL-10-induced immunosuppression seen in human Leprosy or during infection with the *Leishmania major* strain NIH/S, which induces non-healing lesions in infected wild-type mice due to IL-10 production from T<sub>H1</sub> cells<sup>583,584</sup>. Of note, there are viral homologues of IL-10 (vIL-10) that may blunt the immune response to their encoding pathogens<sup>585</sup>. As a result, vIL-10 can prevent the development of cellular immune defenses, increasing the infectivity and survival of the virus that encodes it.

All these different findings demonstrate that the impact of IL-10 is clearly determined by the timing, site of its production, the type of cells that are producing it. To conclude, IL-10 plays a particularly prominent role in curtailing immune-mediated inflammation in the context of infection, allergy, and autoimmunity. In consequence, IL-10 can both impede pathogen clearance and ameliorate immunopathology.

### **Interferon- $\alpha$**

Type I IFNs (IFNs) are hallmark cytokines produced in immune responses to all classes of pathogens<sup>586</sup>. There are nine identified mammalian type I IFN subtypes that include IFN- $\alpha$ , IFN- $\beta$ , IFN- $\epsilon$ , IFN- $\kappa$ , IFN- $\omega$ , IFN- $\delta$ , IFN- $\tau$ , IFN- $\nu$  and IFN- $\zeta$ . With the exception of IFN- $\alpha$ , the rest these subtypes are encoded by single genes. IFN- $\alpha$  includes at 12 distinct proteins encoded by 14 genes<sup>587</sup>. Although many cells in the body are capable of producing type I IFN, plasmacytoid DCs (pDCs) have been labeled the "natural IFN producing cells" due to their distinct molecular adaptations to nucleic acid sensing and capacity to create large amounts of type I IFN<sup>588</sup>. However, recent reports suggest that the cell populations responsible for type I IFN production differ depending on the pathogen, its tissue tropism, and the route of infection<sup>589</sup>. pDCs, monocytes, and other myeloid cells generate type I IFN after activation of the TLR8-MyD88-IRF7 pathway by viral single-stranded RNA<sup>590</sup>. cDCs and macrophages however, mainly produce type I IFN by utilizing the retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs)<sup>591,592</sup>.

Type I IFNs bind to a receptor of two chains that is expressed by virtually all nucleated cells of the body. The type I IFN receptor is composed of two subunits, IFNAR1 and IFNAR2, which are associated with the TYK2 and JAK1, respectively<sup>593</sup>. Following IFNAR engagement by its ligands, canonical type I IFN signaling activates the JAK-STAT pathway, leading to STAT1, STAT2 IFN regulatory factor 3 (IRF-3) and IRF-7 activation and to transcription of IFN-stimulated genes (ISGs)<sup>594</sup>. STAT3 and STAT5 have been also found to be activated by Type I IFNs<sup>595</sup>. Similarly, STAT4 and STAT6 can also be activated by IFN- $\alpha$ , however this appears to be limited to specific cell types, such as endothelial or lymphoid cells<sup>596</sup>. JAK–STAT signaling alone however is not sufficient for the generation of all of the biological functions of

IFNs. Besides the JAK/STAT pathway, type I IFN can also activate other signaling pathways, including the MAPK and PI3K pathways<sup>597,598</sup>. Type I IFNs have also been shown to activate and signal via the NF- $\kappa$ B<sup>599</sup>.

Type I IFNs have been shown influence the function of the immune system through the regulation of cellular homeostasis. Hematopoietic stem cell (HSC) from bone marrow of IFNAR-deficient mice were found to be less frequent and more quiescent compared to wild-type mice, indicating that endogenous IFN-I contribute to the maintenance of HSC through self-renewal<sup>600</sup>. The same report shows that exposure to IFN-I promotes the proliferation of dormant HSC to *in vivo*. These observations suggest that IFN-I may serve as an important signal to re-establish hematopoietic cell homeostasis following infection. Additionally, IFN-I were recently reported to modulate DC turnover<sup>601</sup>. The turnover rate of splenic cDCs is reduced in IFNAR-deficient mice. In cultured IFNAR-deficient bone marrow precursors, the slower turnover of DC was associated with a reduced production of DCs and with a decreased the ability of mature DCs to migrate<sup>601</sup>. Furthermore, IFNAR-deficient cDCs display an increased lifespan and are more resistant to apoptosis *in vitro*<sup>601</sup>. These observations suggest that IFN-I regulate DC homeostasis at two levels: first, by promoting DCs production and mobilization from bone marrow precursors, and second, by enhancing the apoptosis of mature DCs *in situ*. Similarly, IFN-I have been shown to impact T cell homeostasis at multiple levels. Absence of IFN- $\alpha$  impacts T cell function indirectly through the altered homeostasis of DCs. DCs represent the key APCs for the initiation of cell responses and T cells rely on the for *trans*-presentation of IL-15, among other function<sup>602</sup>. Hence, IFN- $\alpha$  acts as an immune adjuvant and promotes maturation of DCs, polarization of TH cells to TH1 cells, and activation of cytotoxic T cells<sup>603</sup>. The utilization of both IFNAR1- and IFNAR2-deficient mice, as well as an IFNAR1-blocking antibody shows that IFN-I also contributes to NK cell homeostasis, activation and antitumor response<sup>604</sup>. Additionally, NK cells cocultured with IFN- $\alpha$ -producing pDCs acquire increased cytolytic capacity. Finally, IFN- $\alpha$  promotes B cell differentiation, antibody production, and Ig class-switching<sup>605,606</sup>.

### **Interleukin-16**

IL-16 was formerly termed lymphocyte chemoattractant factor (LCF), based on its ability to recruit CD4+ T cells<sup>607</sup>. IL-16 is produced primarily by CD8+ T cells but can also be produced by other immune cells such as eosinophils, DCs, monocytes, macrophages, mast cells, as well as activated CD4+ T cells, and non-immune cells like fibroblasts and bronchial epithelial<sup>608</sup>. These cells produce IL-16 in response to stimuli such as mitogens, antigens and or vasoactive amines such as histamine and serotonin<sup>609-611</sup>. IL-16 modulates pathogenic conditions such as asthma and IL-16 is detected in bronchoalveolar lavage fluids from patients with asthma<sup>612</sup>.

IL-16 is unrelated to other cytokines, and its only described receptor is the CD4 molecule. IL-16 is released as a monomer and as a tetramer, which is the primary active form of the molecule<sup>613</sup>. Both tetrameric and monomeric forms are capable of binding to CD4 and can induce chemotaxis and a variety of phenotypic changes in cells expressing this surface glycoprotein<sup>614,615</sup>. Treating CD4+ immune cells with IL-16 results in a cell phenotype that bears a striking resemblance to that seen following anti-CD4+ Treatment<sup>616</sup>.

IL-16 binds to the D4 domain of CD4, which leads to dimerization of CD4 and activation of the CD4-associated src-related tyrosine kinase p56<sup>lck</sup> via autocatalysis<sup>617</sup>. CCR5 can increase IL-16 affinity for CD4 and contributes to activation of p56<sup>lck</sup>, however CCR5 is not an absolute requirement for IL-16 binding and activity<sup>618</sup>. Phosphorylation and activation of p56<sup>lck</sup> results

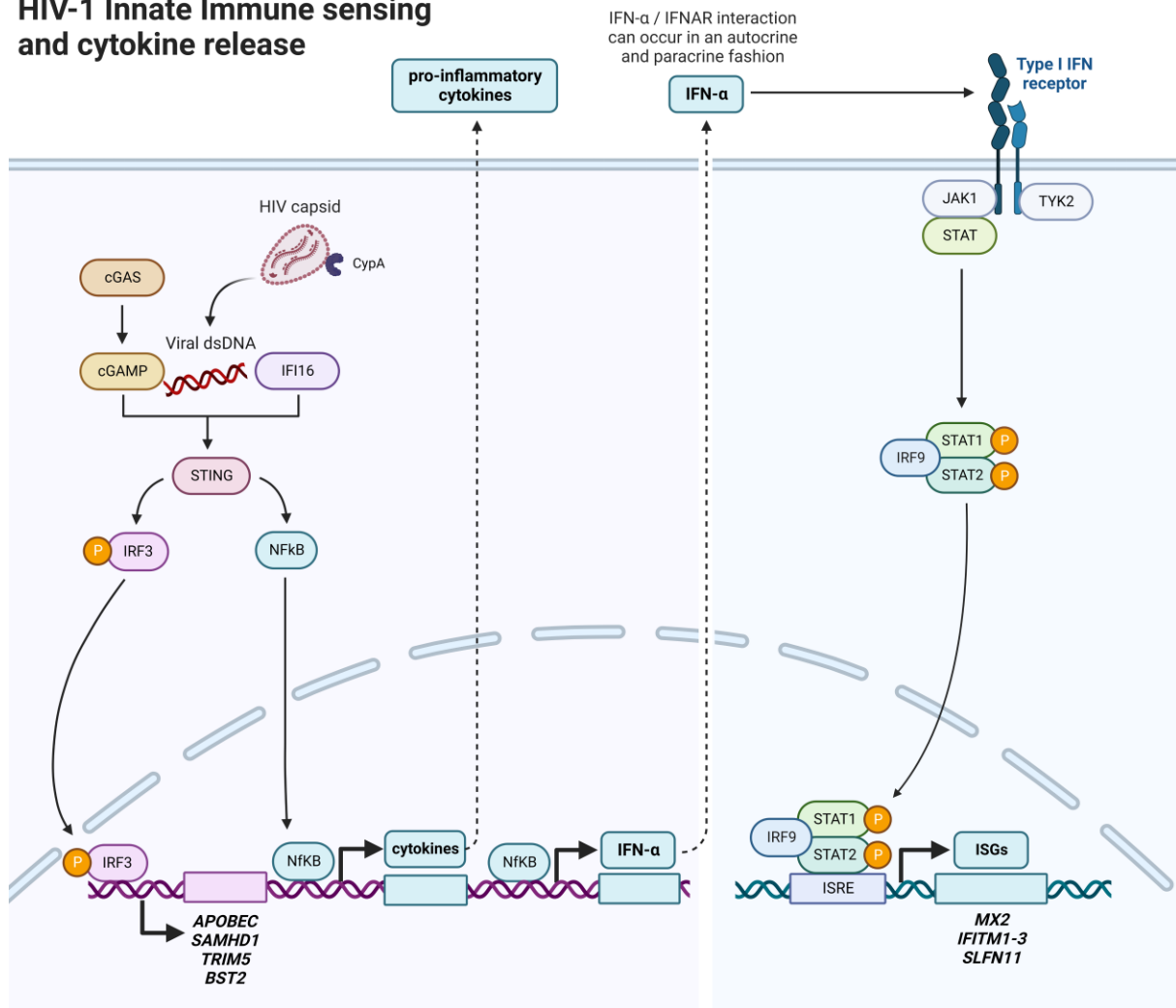
in the initiation of several kinase cascades that mediate cell migration<sup>617</sup>. Protein kinase C (PKC) then translocates to the membrane, activating downstream signaling pathways in target cells, which leads to an increase in intracellular NF- $\kappa$ B, IP<sub>3</sub>, PI3K and calcium flux (Ca<sup>2+</sup>)<sup>616,617</sup>. PI3K activation is required for the chemotactic response to IL-16 in T cells<sup>617</sup>. Other studies have demonstrated the phosphorylation of c-Jun, p38 MAPK and stress-signaling kinase 1 (SEK1) by IL-16 signaling, resulting in activation of stress-activated protein kinase (SAPK) pathway<sup>619</sup>. IL-16 has also been shown to activate STAT6 following interaction with CD4, however downstream signaling and subsequent cellular responses have not yet been extensively identified<sup>620</sup>.

IL-16 mRNA is often present in lymphoid organs, suggesting a role in normal immune function<sup>613</sup>. Upon binding to CD4, IL-16 induces a variety of changes on T cells in addition to chemotaxis. Secreted IL-16 has been reported to play a role in T cell growth. The binding of CD4 by IL-16 stimulates T lymphocytes to upregulate the production of various secreted and surface-bound proteins. Following incubation with IL-16, CD4+ lymphocytes increase the expression of CD25 (IL-2R $\alpha$ ) and CD122 (IL-2R $\beta$ ), leading to proliferation in the presence of IL-2 or IL-15, which indicates that IL-16 is involved in the expansion of T cell populations independent of the recognition of antigen on MHC<sup>614</sup>.

Active forms of IL-16 are stored in granules of CD8+T cells that are the main contributors of this cytokine in an immune response<sup>621</sup>. IL-16 is also a potent chemoattractant not only for CD4+ T cells but for all peripheral immune cells expressing CD4, including monocytes, eosinophils, and DCs<sup>622</sup>. Thus, IL-16 aids in the recruitment of different components of the immune system. IL-16 modulates T cell function by enhancing adhesion and by upregulating the expression of classical MHC class II molecules (primarily HLA-DR) on the membrane of non-stimulated CD4+ T cells *in vitro*<sup>623</sup>. Nevertheless, IL-16 can also have neutralizing effects on T cells function<sup>624</sup>. The interaction of CD4 with the protein-tyrosine kinase p56<sup>lck</sup> and initiation of the tyrosine phosphorylation cascade lead to T cell activation. However, binding of CD4 without subsequent co-receptor signaling can result in incomplete T cell activation, limited IL-2-mediated proliferation, and eventual anergy. Additionally, in the absence of antigen and primed APCs, the effects of an IL-16/CD4 signal results in transient reversible inhibition of subsequent CD3-mediated TCR signaling.

HIV infection is detected in infected cells by two pathogen-recognition receptors (PRRs), interferon inducible protein 16 (IFI16) and cyclic GMP-AMP synthase (cGAS), which recognize viral RT products early during the viral replication cycle. When these sensors interact with viral components, a cell-intrinsic innate immune response is triggered, which drives antiviral defenses and virus restriction. Cell-mediated and soluble factors such as type I and type III interferon, as well as proinflammatory cytokines and chemokines that attract and activate innate immune cells, are also produced during this response (**Figure 15**).

## HIV-1 Innate Immune sensing and cytokine release



**Figure 15. Pathogen-associated molecular patterns (PAMPs) and pathogen-recognition receptors (PRRs) interaction in the innate immune response to HIV-1 infection.**

HIV-1 infection of CD4<sup>+</sup> cells is followed by reverse transcription (RT) of viral RNA into DNA, which yields RT products, including "strong stop" truncation products of RT. These latter molecules, along with RNA-DNA hybrids, are the result of the SAMHD1-induced depletion of nucleoside pools. These products could serve as PAMPs that are sensed by cellular PRRs. The viral capsid, in association with the chaperone protein CypA, protects RT products from detection by PRRs; however, mutations that change the CA-CypA interactions expose the HIV-1 DNA products, allowing PRRs in the cytosol to detect them. IFI16 and cGAS sense and bind to HIV-1 DNA in the cytosol. Activated IFI16 facilitates direct STING binding or signals to STING via an intermediate whereas cGAS produces a cGAMP second messenger that binds to the STING adapter protein. STING is activated to engage signaling cofactors that activate the IRF3 and NF- $\kappa$ B transcription factors, resulting in the expression of target genes such as HIV-1 restriction factors, type I IFNs, and proinflammatory cytokines. Infected cells and bystander cells respond to IFNs by binding to the IFN- $\alpha/\beta$  receptor, which causes signaling through the Jak-STAT pathway and the transcription factors STAT1, STAT2 and IRF9 to promote the expression of ISGs.

## Cytokines in HIV infection

Many reports demonstrated the association between cytokines produced during the acute phase of HIV/SIV infection and clinical outcomes. For instance, elevated plasma IL-7 and IL-15 levels were found to be deleterious and correlate with higher viral set points during early HIV infection, whereas plasma IL-12 and IFN- $\gamma$  levels appear beneficial and correlate with lower viral set points<sup>625</sup>. Early IL-10 upregulation has also been shown to play a protective function in studies, with polymorphisms resulting in greater IL-10 production and lower viral set points<sup>626</sup>. These human studies imply that cytokine profiles during acute infection influence the viral set point, and non-human primate studies support this conclusion<sup>627,628</sup>. A number of pro-inflammatory and anti-inflammatory cytokines can be detected in individuals with symptomatic acute infection<sup>629,630</sup>. Cytokines that are produced by cells of the innate immune system are the first to be detected. IFN- $\alpha$  and IL-15 are among the first cytokines to be detected within 5 days after detection of viremia<sup>32</sup>. IL-15 and other pro-inflammatory cytokines have been associated with a higher viral set point in SIV infection<sup>628</sup>. IL-7 was identified as a danger signal during acute SIV infection<sup>631</sup>. IFN- $\alpha/\beta$  correlated with high viral loads and rapid disease progression or onset of AIDS<sup>632</sup>. IL-10 secretion, which shortly follows that of pro-inflammatory cytokines during primary infection, is associated with premature induction of immunosuppression<sup>633</sup>. IL-10 was also recently proposed to contribute to reservoir establishment and persistence in SIV infected macaque<sup>634</sup>. Interestingly, IFN- $\alpha$ , and IL-10 mRNA and protein levels are increased earlier in non-pathogenic SIV infection compared to pathogenic SIV infection<sup>635</sup>. However, it was found that although natural hosts of SIV upregulate cytokines such as IFN- $\alpha$  and IFN- $\gamma$ , these cytokines are downregulated much faster, and the production of anti-inflammatory cytokines occurs earlier in non-pathogenic SIV infection, indicating that these cytokines may play a crucial role in limiting SIV pathogenesis<sup>32,636</sup>.

Given that a significant increase in HIV viral load occurs at the same time as the large burst of cytokines during acute HIV infection, cytokines could favor the establishment of HIV reservoir<sup>637</sup>. Several cytokines that are released during the acute phase are known to upregulate HIV expression and render CD4+ cells more permissive to HIV<sup>638,639</sup>. On the other hand, the reservoir of long-lived infected cells may emerge as a consequence of the development of the anti-inflammatory response. By limiting the availability of cellular transcription factors required for active viral gene expression, repressive signals, particularly those induced by anti-inflammatory cytokines, may enable the generation of quiescent memory cells containing HIV integrated DNA and therefore the establishment of viral latency in long-lived memory CD4+ T cells.

Effects of cytokines on HIV can be either inhibitory, stimulatory, or bifunctional (that is both inhibitory and stimulatory), indicating that HIV infection and replication *in vivo* and *in vitro* can be under the continuous regulation of a complex cytokine network produced by a variety of cells. The susceptibility of cells to productive HIV infection depends on the level of T-lymphocyte activation and the stage of monocyte/macrophage differentiation, suggesting that cytokines and growth factors that promote activation and differentiation of cells of the immune system can also modulate both HIV infection and replication<sup>640-642</sup>. *In vitro*, several cytokines have been reported to increase HIV infection. This includes IL-1 $\beta$ , IL-2, IL-3, IL-6, IL-7, IL-12, IL-15 to cite a few<sup>643-645</sup>. IFN- $\alpha$ , IFN- $\beta$  and IL-16 have been primarily described as suppressors of HIV expression<sup>646-648</sup>.

Other cytokines such as IL-4, IL-13, IL-10, and IFN- $\gamma$ , have been found to reduce or enhance viral replication depending on the infected cell type and the culture conditions<sup>649–653</sup>. Many cytokines such as IFNs can influence HIV replication in both CD4+ T cells and macrophages, while others are lineage specific. In addition, the effect of a specific cytokine can be influenced by the presence of other cytokines. Certain cytokines will exert a synergistic<sup>372,654–656</sup> effect and other cytokines will have an antagonistic effect<sup>657–660</sup> in regulating HIV replication. CC-chemokines can also exert dichotomous effect on the replication of T-tropic versus M-tropic HIV strains.

HIV infection causes chronic immune activation, which in turn causes the cytokine network to be dysregulated. Many of the observed alteration in cytokine release contribute to HIV pathogenesis by enhancing viral replication, silencing effector cells of the immune system and decreasing its ability to mount an efficient antiviral response, as well as by causing cytokine-mediated cytopathic sequelae.

### **Interleukin-2**

IL-2 is a potent enhancer of HIV replication, partially due to the dependence of HIV replication on T cell proliferation<sup>21,661</sup>. It was also suggested that IL-2-induced upregulation of HIV replication is mediated predominantly through a post-transcriptional mechanism independent of direct HIV LTR activation<sup>662</sup>. Although IL-2 appeared to have an enhancing effect in activated CD4+ T cells but not in unstimulated cells, other studies have shown that IL-2 increases susceptibility of resting CD4+ T cells to HIV, with minimal impact on T cell activation<sup>663</sup>. IL-2 can also increase the expression of CCR5 on CD4+ T cells<sup>664</sup>. Whereas, in macrophages, it has been shown to impede HIV replication by suppressing the expression of CD4 and CCR5<sup>665</sup>. IL-2 also induces the production of cytokines that also enhance HIV replication, and neutralization of these proinflammatory cytokines results in viral replication suppression<sup>666</sup>. IL-2 also inhibits apoptosis induced by cross-linking of gp120 and CD4 receptor in CD4+ T cells<sup>667,668</sup>. IL-2 stimulation maintains the expression of Bcl-2 protein levels, decreases induced apoptosis in CD4+ T cells and supports the expansion of CD4+ T cell.

IL-2 *in vivo* administration in PLWH induced transient blips of HIV viremia<sup>669</sup>. Phase III trials with IL-2 demonstrated that IL-2 used either alone or in combination with an anti-CD3 antibody or with IFN- $\gamma$  failed at eradicating HIV and did not translate into meaningful clinical benefit<sup>670–673</sup>.

### **Interleukin-7**

IL-7 treatment of CD4+ T cells results in a significant increase in HIV production<sup>674</sup>. IL-7 enhances HIV replication in resting CD4+ T lymphocytes, at least in part by inducing phosphorylation of SAMHD1 and improving reverse transcription<sup>675</sup>. Furthermore, studies have indicated that IL-7 contributes to the maintenance of HIV reservoir in T cells<sup>675</sup>. A molecular basis for this has recently been proposed; involving firstly mTOR-mediated enlargement of the dNTP pool available for viral reverse transcriptase activity, via coordinated increases in the activity of nutrient uptake and nucleotide biosynthesis pathways, and secondly, increased acetyl-CoA abundance, which results in the acetylation and stabilization of microtubules, facilitating cytoplasmic transport of viral products<sup>365</sup>. Importantly, the inhibition of both PI3K signaling and GLUT1 abrogated this effect<sup>362,363</sup>. IL-7 can also regulate HIV transcription and induce reactivation, through interaction with IL-7R and the NF-AT<sup>676</sup>.

Moreover, IL-7 was significantly more effective in enhancing HIV proviral reactivation in CD8<sup>+</sup>-depleted PBMCs than either IL-2 alone, or IL-2 combined with PHA<sup>674,677</sup>.

However, recent research, suggest that IL-7 enhances viral production in productively infected cells but does not disrupt viral latency in latently infected cells<sup>678,679</sup>. IL-7 has been linked to enhanced CXCR4, but not CCR5, expression and is believed to aid in the R5 to X4 switch observed in the late stages of HIV infection<sup>680</sup>.

In addition, IL-7 plays a role in early T cell development and proliferation. It can regulate Bcl-2 expression and has been shown to promote both CD4<sup>+</sup> and CD8<sup>+</sup> T cell survival during HIV infection<sup>681,682</sup>. IL-7 increases the number of circulating CD4<sup>+</sup> and CD8<sup>+</sup> T cells, predominantly of central memory phenotype. However, both CD4<sup>+</sup> and CD8<sup>+</sup> T cells have reduced CD127 (IL-7R $\alpha$ ) expression during chronic HIV infection, which impairs IL-7 responsiveness, cell survival, and cytotoxic function<sup>683</sup>. It is likely that this is reflected by the reduced potential of homeostatic recovery of the remaining T cell pool in PLWH, which all contribute to pathogenesis. Hence, several studies argue that IL-7 therapy in PLWH is beneficial in restoring T cells numbers after ART.

*In vivo* administration of IL-7 in virally suppressed PLWH increased the numbers of CD4<sup>+</sup> T and CD8<sup>+</sup> T cells as well, which has been linked to increased mTOR activity in HIV-specific CD8<sup>+</sup> T cells<sup>681,682,684,685</sup>. The same studies reported the occurrence of viral blips, particularly in those individuals who received the highest doses of IL-7<sup>681,682</sup>. Levy and colleagues show that the administration of IL-7 to virally suppressed individuals results in increased total levels of cell associated HIV DNA, proportional to CD4<sup>+</sup> T cell expansions<sup>686</sup>. These data imply that IL-7 may increase or at least sustain the pool of persistently infected cells during ART, which is consistent with the discovery that IL-7-induced homeostatic proliferation of central memory latently infected CD4<sup>+</sup> T cells fails to effectively reactivate HIV production and clear the infected cells<sup>687</sup>.

### **Interleukin-10**

Generally, IL-10 is not regarded as a major contributor to T cell development. It is however considered a prototypical anti-inflammatory cytokine, significantly contributing to the maintenance and reestablishment of immune homeostasis. . Furthermore, IL-10 expression in B cells and macrophages, which was induced by SIV infection, has been correlated with immunosuppression in non-human primates<sup>688</sup>. IL-10 is one of the signature cytokine that is characteristic of the immunosuppressive phase that emerges after the proinflammatory phase in acute HIV infection. IL-10, a component of the T<sub>H2</sub>-type immune response, may prevent HIV from replicating *in vivo*, potentially by reducing the proliferation of T<sub>H1</sub> cells, inhibiting macrophage division, and secreting less pro-inflammatory cytokines. Curiously, an increased susceptibility to HIV infection is linked to IL-10 gene mutations<sup>626</sup>. SNP in the promoter of IL-10 gene results in either high (C) or low (A) alleles. Carriers of A alleles are thought to have an increased risk of HIV infection, and PLWH with a A/A genotype progress quicker to AIDS than those who are homozygous for the high producer allele (C/C). The C/C genotype is present in around 25–30% of individuals with a long latency in the course of AIDS (more than 10 years)<sup>689</sup>.

IL-10 was among the first cytokines that have been described to suppress HIV replication in macrophages and was found to have modest anti-HIV activity in CD4<sup>+</sup> T cells<sup>690,691</sup>. One possible mechanism for the anti-HIV activity of IL-10 may be downregulation of cytokine production (such as TNF- $\alpha$ , GM-CSF, or IL-6). IL-10 induced HIV inhibition could be reversed



by the addition of TNF- $\alpha$ <sup>692</sup>. However, the inhibitory effect of IL-10 was also observed with lower IL-10 concentrations than those required to suppress TNF- $\alpha$  production<sup>691</sup>. After HIV inoculation, primary macrophages treated with IL-10 exhibit an increase of Gag protein, which is indicative of an inhibitory effect at the level of viral assembly<sup>693</sup>. It is also likely that IL-10 acts on a transcriptional level, which could also explain the inhibitory effect on other cytokine production. Given that the inhibitory effects of IL-10 were more pronounced in acute infection models, it should not be excluded that this cytokine could also affect early steps of the replication cycle<sup>694</sup>. Paradoxically, there are studies demonstrating that IL-10 used at lower concentrations, synergizes with stimulatory cytokines to enhance HIV production in monocyte-derived macrophages<sup>695</sup>. Overall, IL-10 appears as a cytokine that can have complicated regulatory effects on HIV expression depending on its concentration and the presence of other HIV regulatory cytokines.

HIV transcription can be inhibited by IL-10 by limiting T cell activation, but it can also dampen virus-specific immunity, which may facilitate viral persistence<sup>560,696</sup>. In addition to its immunomodulatory effect, IL-10 may directly contribute to HIV latency by inhibiting activation and viral production in infected CD4+ T cells. Evidence of increased IL-10 protein expression and transcriptomic signatures in lymphoid tissues during chronic and ART-treated SIV infections is suggestive of a role for IL-10 in maintaining a pool of target cells in these sites that are known niches for viral persistence<sup>634,697</sup>. Together, these findings imply that IL-10 may play a role in viral persistence, however the possible involvement of IL-10 in the development of the latent viral reservoir has yet to be explicitly confirmed.

Chronic HIV infection results in significant increase of plasma levels of IL-10 when compared to HIV-seronegative individuals and HIV controllers<sup>698</sup>, thus reducing the capacity of the immune system in PLWH to eliminate the virus. In addition to controlling cytokine production, IL-10 also promotes the expression of PDL-1 on macrophages, which when engaged with the PD-1 receptor on T cells during antigen presentation, can decrease T cell activity<sup>699</sup>. Increased IL-10 secretion and mRNA expression has been found in PBMCs obtained from PLWH at all stages of HIV infection compared to HIV-negative<sup>700-702</sup>. In persons with advanced HIV infection, elevated IL-10 production correlates with increased levels of the pro-inflammatory cytokines<sup>703</sup>. IL-10 appears to play a major role in the viral escape from the immune system<sup>560</sup>. Accordingly, several HIV-viral proteins can induce the production of IL-10, including Tat<sup>704-706</sup>.

The observation of the inhibitory effects of IL-10 on HIV replication sparked the interest in its combined utilization with ART. Although the inhibition of HIV replication by IL-10 has been observed *in vitro*<sup>707</sup>, the administration of IL-10 to people with chronic infection resulted in little or no impact on viral replication<sup>708</sup>.

### **Interleukin-15**

IL-15 is among the first cytokines elevated upon HIV infection. Elevated IL-15 is associated with higher plasma viral load during acute HIV infection<sup>625</sup>. In contrast, IL-15 levels are decreased significantly in the sera of people with chronic infection. These low levels of IL-15 increase in response to effective ART<sup>709</sup>. IL-15 is produced by APCs including monocyte/macrophages and DCs. IL-15 is expressed at higher levels in monocytes from HIV-long-term non-progressors compared to progressors or seronegative controls<sup>710</sup>.

There are contrasting reports of either no impact or enhancement of IL-15-induced HIV replication and the effect seems to depend on HIV-producing target cell, activation status,

concentration of IL-15 as well as the stage of infection<sup>644,662</sup>. Early reports show that IL-15, to a lesser degree than IL-2, upregulates HIV replication in PBMCs and in T cell lines *in vitro*<sup>645,662,711</sup>. IL-15 also enhances HIV production in PBMCs obtained from PLWH. This enhancement was independent of direct HIV LTR activation, cellular proliferation or other cytokines production<sup>712</sup>. Moreover, as mentioned previously for IL-2 and IL-7, IL-15 can also increase the susceptibility of resting CD4+ T cells to HIV, with minimal impact on T cell activation. It has been proposed that IL-15 causes phosphorylation of SAMHD1 via JAK1 induction, which contributes to more important dNTP pool favoring HIV reverse transcription<sup>713</sup>.

IL-15 may counteract the pro-apoptotic signals present in infected CD4+ T cells and enhance the creation and maintenance of long-lived latently infected cells as it encourages the development and maintenance of memory T cells through the upregulation of Bcl-2 expression<sup>126</sup>, which is involved in HIV latency<sup>714</sup>. Several studies have linked IL-15 with increased viral set point<sup>715</sup>, which could also impact reservoir seeding.

Treatment with IL-15 during acute SIV infection did not decrease the viral load, instead it altered the establishment of the set point<sup>628,716</sup>. This suggest that IL-15 could influence the dynamics of SIV replication during acute infection<sup>717</sup>. Contradictory results concerning the impact of IL-15 on immune reconstitution in chronically SIV-infected macaques highlight the importance of the timing for IL-15 administration and secretion during the course of infection<sup>716,718,719</sup>. Similar to IL-7, IL-15 is important for the homeostatic expansion of T lymphocytes. As a result, it may have both favorable and unfavorable effects on HIV infection and pathogenesis.

### **Interleukin-16**

IL-16 is a natural ligand for the CD4 receptor. IL-16 levels in plasma have been reported to increase upon HIV infection. A decrease in IL-16 serum levels is associated with the development of AIDS whereas asymptomatic PLWH have serum levels of IL-16 that are within the normal range or slightly elevated, when compared to HIV-negative individuals<sup>720,721</sup>. It has been shown to decrease HIV replication *in vitro*, in CD4+ T cells, MDMs and DCs<sup>722-724</sup>. However, this is not due to steric inhibition of viral binding to T cells since IL-16 binds to CD4 at an epitope unique from that of HIV. However, it has been suggested that IL-16 may signal through CCR5<sup>725</sup> and/or CXCR4, which may be also expected since CD4 and CCR5 as well as CD4 and CXCR4 are constitutively associated<sup>726</sup>. Pretreatment of macrophages with IL-16 was found to inhibit both R5 and R5/X4 HIV replication in primary macrophages at the level of entry<sup>722</sup>, most likely as a result of desensitization of the receptors needed for HIV entry<sup>725</sup>. In addition, IL-16 appears to have a suppressive impact at the transcriptional regulatory level. It was shown that IL-16 pretreatment inhibited both PMA- or Tat-stimulated HIV promoter activity in transient transfection studies using HIV LTR-reporter gene constructs<sup>724</sup>. This impact was not simply attributable to decreased binding activity of transcription factors like NF- $\kappa$ B indicating that IL-16 stimulation engages a transcriptional repressor.

### **Interleukin-21**

The identification of IL-21 is relatively recent and its impact on HIV infection has been least explored within the  $\gamma$ -cytokine family. IL-21 plasma levels have been shown to decrease during primary HIV infection, only partially recovered upon initiation of ART, and preserved in EC<sup>727</sup>. IL-21 can reduce HIV infection *in vitro* of lymphoid CD4+ T cells. This effect appears to be

mediated by enhancing cell-intrinsic resistance to the virus, silencing HIV transcription and promoting latency through the induction of microRNA-29<sup>728</sup>.

IL-21 increased intestinal T<sub>H17</sub> cells reconstitution in SIV-infected rhesus macaques on ART, with decreased microbial translocation, lower immune activation in both the gut and the circulation. Immune activation and plasma viral loads remained reduced after ART interruption, showing that IL-21 therapy had a greatly positive effect<sup>729,730</sup>.

### **Interferon- $\alpha$**

IFN- $\alpha$  is among the first cytokine elevated after detection of viremia. The innate immune response to viral infection depends on it. Alternatively, type I (IFN- $\alpha$  and IFN- $\beta$ ) and type II (IFN- $\gamma$ ) IFNs are typically regarded as antiviral cytokines and they interfere with viral replication. IFN- $\alpha$  can block HIV reverse transcription at several levels, leading to reduced virus production<sup>731</sup>.

IFN- $\alpha$  inhibits HIV replication at multiple levels of the viral cycle in both activated CD4+ T cells and macrophages. The predominant level of blockade seems to occur prior to formation and integration of the provirus by suppressing reverse transcription<sup>732</sup>. IFN- $\alpha$  enhances RfFs such as APOBEC/TRIM proteins, which can potentially prevent reverse transcription and viral replication<sup>733</sup>. IFN- $\alpha$  has been also shown to prevent transcription of integrated provirus<sup>734</sup>. In chronically infected cell lines, IFN- $\alpha$  can block viral assembly and release<sup>735</sup>. The results of several clinical trials support a predominantly antiviral activity when IFN- $\alpha$  is administered to PLWH in the absence of ART<sup>736,737</sup>. IFN- $\alpha$  was proven capable of suppressing HIV replication, even in the absence of ART, likely through enhanced innate immune effectors or HIV-specific CD8+ T cells. The effects of IFN- $\alpha$  after ART-mediated immune restoration, tested in recent clinical trials suggest an enhanced control of HIV replication and decreased HIV integration than when only ART is used<sup>738,739</sup>. These findings suggest that IFN- $\alpha$  may help reduce the size of cellular reservoir<sup>738</sup>.

An *in vivo* investigation of the effects of IFN-I inhibition in SIV-infected rhesus macaques revealed that IFN-I does have a major impact on SIV natural history and replication<sup>740</sup>. In that work, the investigators used an IFN receptor antagonist to prevent all IFN-I subtypes from signaling prior to SIV infection. They observed that blocking IFN-I during the early stages of infection resulted in significantly higher viral loads and faster CD4+ T cell decline during the chronic phase of infection, which was associated with faster progression to AIDS in the IFN-I-impaired animals, despite a reduction in activation markers on lymphocytes. Moreover, IFNAR blockade in SIV-infected rhesus macaques during acute infection leads to accelerated mortality<sup>740</sup>.

Despite IFN-I antiviral properties, numerous lines of data imply that continuously high levels of IFN-I production are associated with long-term immune activation during chronic HIV/SIV infection<sup>648</sup>. Nonpathogenic SIV infection involves downregulation of IFN-I production and upregulation of ISG during the chronic phase of infection<sup>635,741</sup>. Furthermore, exogenous IFN- $\alpha$  (as in the treatment of hepatitis C virus infection) has an anti-proliferative consequence on lymphocytes, indicating that IFN-I may have a negative impact on T cell homeostasis in the setting of a chronic, persistent viral infection, such as HIV<sup>742,743</sup>.

It is important to note that there are various types of type I IFN. Differences observed between the studies mentioned above can be due to difference in types of IFN used.

## Cytokines in cellular metabolism

As previously stated, cellular metabolism is central in the orchestration of the immune response, and metabolism plays also a key role in regulating cytokine release upon infection<sup>324,744–748</sup>. As activated immune cells have large metabolic needs to maintain their immunological activity, they are extremely vulnerable to the metabolic properties of their microenvironment. Reduced availability of oxygen, glucose, and amino acids in the tumor microenvironment, for example, can impede T cell function; however, the accumulation of metabolic intermediates and end-products in malignant and inflamed tissues also affects T cell activity<sup>749,750</sup>. In addition to being dependent on the metabolic characteristics of their surroundings, immune cell metabolism is heavily influenced by factors present in altered abundance in pathology, either systemically or locally, in diseased tissue. Cytokines and their signaling pathways are widely established to have powerful impacts on T cell activation, differentiation, and function. However, the role of cytokines in T cell metabolism, particularly in disease, is less understood. Some studies have begun to investigate the possible involvement of cytokine-mediated metabolic alterations in modulating T cell activity, but further in-depth research is needed to understand the mechanisms and clinical implications of these effects. The focus of this section, as well as a major part of my PhD work, is to understand how T cell metabolism can also be controlled by the activity of cytokines secreted in response to HIV infection. Because of their importance in T cell homeostasis, most studies in this field have focused on  $\gamma$ c cytokine family, which includes IL-2, IL-7, IL-15, and IL-21<sup>751</sup>.

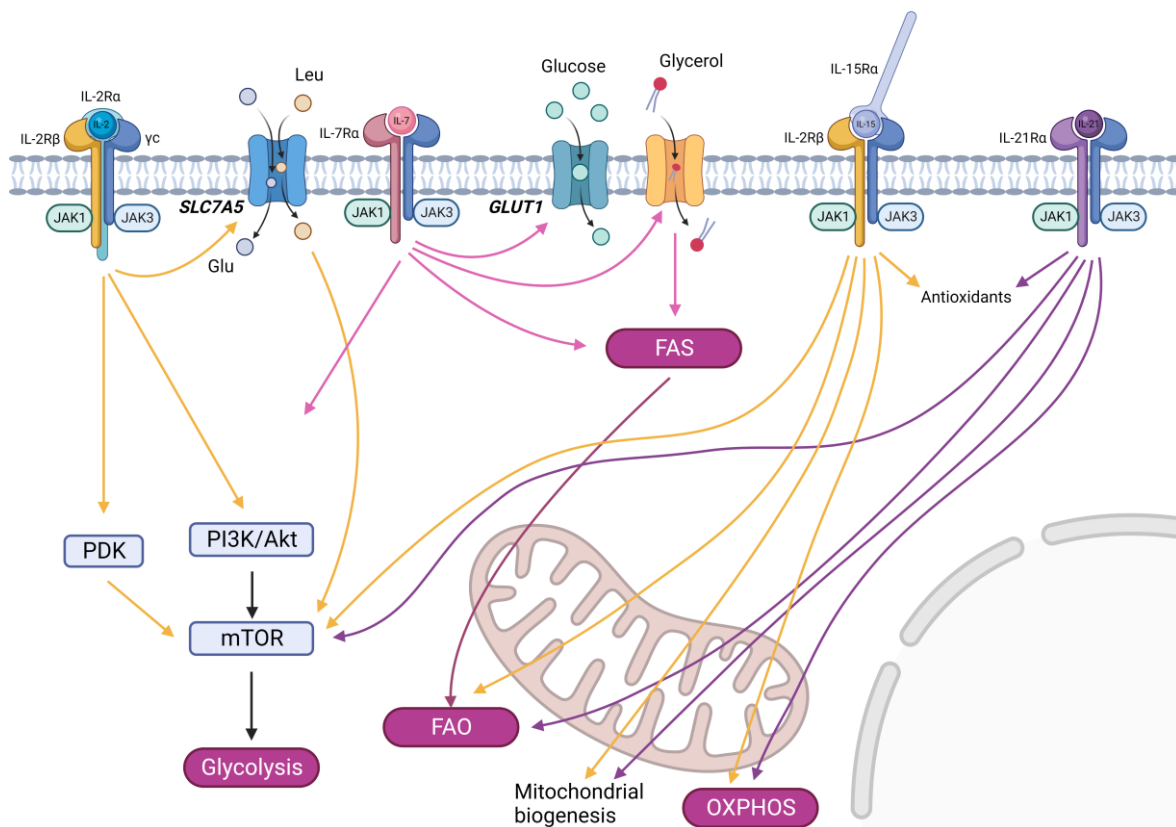
Each of these cytokines has a distinct role in controlling the metabolic changes necessary at various stages of a T cell response. The effects of  $\gamma$ c cytokines are generally coordinated through the activation of three major signaling pathways: PI3K/Akt, JAK/STAT, and MEK/ERK<sup>751</sup>. Both cytokines and JAK/STAT signaling are known to be involved in cellular metabolism (**Figure 16**). The induction of SLC1A5 and SLC3A2/SLC7A5 expression by IL-2 and IL-15 via the JAK3/STAT5 pathway exemplifies how cytokines act at the frontier of immune response and cellular metabolism<sup>752–754</sup>. JAK/STAT signaling pathway has directly been linked to glucose, lipid metabolism as well as mitochondrial function. For instance, Akt and STAT5 have been shown to activate processes associated with cellular metabolism, such as by increasing glycolysis during the later stages of TCR and co-stimulatory receptor activation<sup>755</sup>. Further to that, the JAK/STAT pathways have been linked to a variety of metabolic diseases<sup>756–760</sup>. JAK/STAT signaling has been shown to be highly engaged in metabolically active tissues such as adipose, liver, skeletal muscle, and pancreatic cells, as well as metabolically-demanding immune cells such as macrophages, NK cells, and T cells. An increasing amount of evidence suggests that most STAT proteins are also located in mitochondria<sup>761</sup>. Mitochondrial STAT3 in particular has been closely linked to mitochondrial function and cellular ATP levels<sup>762</sup>. The impact of mitochondrial STAT proteins on whole-body metabolism and glucose homeostasis has yet to be investigated, highlighting this as an important area for future research.

The mechanism of action of SOCS proteins provides more evidence for JAK/STAT and cytokines involvement in cellular metabolism. SOCS proteins are a group of inhibitors of cytokine signaling<sup>763</sup>. To prevent excessive inflammatory responses, SOCS1 negatively regulates a number of glycolytic enzymes, such as hexokinase, LDHA and GLUT1, in macrophages during sepsis<sup>764</sup>. In this sepsis model, macrophage metabolic reprogramming is

dependent on the STAT3/HIF1 $\alpha$ /glycolysis axis and inhibition of glycolysis ameliorates susceptibility to sepsis.

### **Interleukin-2**

Because certain amino acids are essential for mTOR induction, the expression of corresponding nutrient transporters is critical for enabling appropriate nutrient uptake and efficient mTORC1 activation<sup>765,766</sup>. It has been shown that upregulation of both SLC7A5 and the glutamine-transporter SLC1A5 is essential for mTORC1 activity<sup>346,765,767,768</sup>. These transporters allow amino acids such as leucine to enter the cell, activating nutrient-sensing Rag GTPases, which are enzymes upstream of mTORC1<sup>344,345,768,769</sup>. Pro-inflammatory cytokines that activate lymphocyte effector functions tend to increase the expression of proteins involved in AA transport, such as SLC1A5, SLC3A2, and SLC7A5. For example, IL-2 increased the expression of SLC1A5 and SLC3A2/SLC7A5 (ASCT2) transporters in NK cells, which are required for IFN- $\gamma$  generation and degranulation<sup>752</sup>. In T cells, IL-2 was demonstrated to boost SLC7A5 expression, which was sustained with continued cytokine exposure<sup>305,770,771</sup>.



**Figure 16. T cell metabolism and cytokine signaling.**

IL-2 coupled to the IL-2 receptor (IL-2R) can activate mTOR via PDK and PI3K/Akt in CD8<sup>+</sup> and CD4<sup>+</sup> T cells, respectively. IL-2 stimulates the expression of SLC7A5 at the cell surface, resulting in the intake of amino acids and further activation of mTOR and glycolysis. To maintain glycolysis, IL-7 also signals via the PI3K/Akt/mTOR pathway and promotes GLUT1 expression. IL-7 promotes fatty acid oxidation by increasing glycerol uptake and triacylglycerol production in CD8<sup>+</sup> memory T cells. In T cells, IL-15 and IL-21 promote antioxidant synthesis, fatty acid oxidation, mitochondrial biogenesis, and oxidative phosphorylation. Both IL-15 and IL-21 have been demonstrated to boost mTOR activity in CD8<sup>+</sup> memory T cells and T<sub>REGS</sub>. Additionally, IL-1 $\beta$  stimulates mTOR activity and glycolysis in CD4<sup>+</sup> T cells, favoring the T<sub>H17</sub> cell phenotype. TGF- $\beta$ , on the other hand, suppresses glycolysis in CD4<sup>+</sup> T cells via the PI3K/Akt/mTOR pathway, as well as OXPHOS in CD4<sup>+</sup> memory T cells (adapted from Bishop EL, et al., *Front Immunol.* 2021<sup>905</sup>).

Moreover, it has been demonstrated that blocking these food transporters has a deleterious influence on the effector functions of NK and T cells. SLC3A2 deletion, for example, hinders T cell expansion, whereas SLC7A5 loss prevents T cell effector differentiation, mTORC1 activation, and c-Myc expression<sup>305,772,773</sup>. SLC7A5 deletion also reduced CD4+ T cell expansion and the release of certain proinflammatory cytokines<sup>773</sup>. Furthermore, SLC7A5 and SLC1A5 deficient mice show impaired metabolism and mTORC1 activation<sup>346,774</sup>. Finally, pharmacological inhibition of SLC1A5 and SLC3A2 impaired NK cell effector functions, while inhibition of SLC7A5 in cytokine-activated NK cells resulted in lower c-Myc protein levels and mTORC1 signaling<sup>752,775</sup>. Along these lines, glutamine and  $\alpha$ -KG have been shown to govern IL-2-induced transcriptome alterations, in part through changes in DNA and histone methylation states and CCCTC-binding factor (CTCF) genomic binding<sup>776</sup>. Upon TCR engagement, IL-2 is essential for upregulation of glucose metabolism. IL-2-induced mTOR increases glycolysis through activation of Pi3K/Akt signaling to promote T<sub>H1</sub> differentiation<sup>777,778</sup>. IL-2 promotes aerobic glycolysis, robustly inducing lactate dehydrogenase LDH and lactate production<sup>779</sup>.

### **Interleukin-7**

Similar to IL-2, stimulation of T lymphocytes with IL-7 increases the transcription of several amino acids transporters, including SLC1A4, SLC1A5 and SLC7A5<sup>774,780</sup>. IL-7 is also essential for T cell glycolysis homeostasis because it regulates GLUT1 and HK2 expression<sup>781,782</sup>. TSCM cells display significant levels of IL-7-induced GLUT1 expression but low levels of lactate export, showing that pyruvate is preferentially oxidized in the mitochondria, implying that IL-7 may also play a function in the OXPHOS pathway<sup>783</sup>. IL-7 also improved the long-term survival of CD8+ memory T cells by increasing glycerol uptake for triglyceride synthesis and storage, which provides a substrate for FAO<sup>784</sup>.

### **Interleukin-15**

Expression of amino acids transporters SLC1A5, SLC7A5, and SLC3A2 was increased in NK cells treated with IL-15<sup>754</sup>. In CD8+ T cells, IL-15 promotes mitochondrial fusion and enhances the mitochondrial spare respiratory capacity (SRC). This is linked to an increase in CPT1A, which catalyses the transfer of an acyl group from coenzyme A to l-carnitine, thereby promoting FAO<sup>328,785</sup>. In addition to these mitochondrial changes, IL-15 promotes the expression of antioxidant molecules in CD8+ T cells such as glutathione reductase, thioredoxin reductase 1, peroxiredoxin, and superoxide dismutase, which have been linked to increased longevity *in vivo*, most likely by mitigating the increased levels of ROS generated by increased mitochondrial OXPHOS activity<sup>786,787</sup>. IL-15 has also been shown to promote mTORC1 activity in CD8+ T cells<sup>788</sup>.

### **Interleukin-21**

IL-21, like IL-15, has been shown to promote FAO over glycolysis, mitochondrial biogenesis and fitness, and antioxidant production in T cells<sup>779,789</sup>. IL-21 has also been shown to upregulate both mTORC1 and mTORC2 and the blockage of mTOR using rapamycin corrects T<sub>REG</sub> cell function in systemic lupus erythematosus (SLE)<sup>790</sup>.

### **Interleukin-10**

Recent data demonstrated that metabolic reprogramming of terminally exhausted CD8+ T cells, which displayed a dysregulated metabolism, by IL-10 efficiently enhances anti-tumor

immunity<sup>791</sup>. IL-10 was also shown to mediate metabolic reprogramming in macrophages<sup>792</sup>. IL-10 promotes insulin sensitivity and oxidative phosphorylation<sup>791,793</sup>. Additionally, IL-10 was found to impede glycolysis switch by inducing the expression of an mTOR inhibitor, DNA damage-inducible transcript 4 (DDIT4), to prevent glucose uptake<sup>792</sup>.

### **Interferon- $\alpha$**

In general, type I IFNs appear to stimulate glycolysis. In mouse embryonic fibroblasts, for example, IFN induces a PI3K/AKT-dependent glucose uptake, which may increase ATP production<sup>794</sup>. The involvement of glycolysis appears to be significant in mitochondrial fielding, and there are also noteworthy situations where type I IFN increases oxygen consumption. Through an autocrine loop, mouse pDCs increase oxidative phosphorylation and ATP generation 24 hours after stimulation with poly(I:C) or promptly after type I IFN treatment<sup>795</sup>. Nonetheless, influenza virus activation of human pDCs resulted in Warburg-like remodeling of energy metabolism, including increased glycolytic flux and reduced mitochondrial respiration<sup>796</sup>.

According to new findings, a key antiviral mechanism of type I IFN appears to be the suppression of cholesterol and fatty acid biosynthesis derived from glucose<sup>797,798</sup>. Inhibiting the production of cholesterol has a direct antiviral impact. SREBP2 and SREBP1 are the primary transcription factors involved in the control of the sterol biosynthesis pathway<sup>799</sup>. IFNs effectively decrease SREBP2 transcription and expression via IFNAR1<sup>797</sup>. Surprisingly, just restricting cholesterol synthesis stimulates spontaneous type I IFN production and improves antiviral immunity<sup>798</sup>. FAO is also known to be induced by IFNs. Lipid-activated nuclear receptor peroxisome proliferator-activated receptors (PPARs); such as PPAR- $\delta$ , stimulate JAK/STAT signaling and, as a result, boost the IFN response<sup>334,335</sup>. FAO furthermore offers antiviral elements. Oxysterols, which are generated from cholesterol, are key systemic mediators that control various immune activities. In macrophages, IFNs or viral infections induce and secrete one oxysterol: 25-hydroxycholesterol (25-HC)<sup>800</sup>. 25-HC is a soluble antiviral factor produced from cholesterol via STAT1 activation of cholesterol-25-hydroxylase in response to IFN. Several prior investigations have previously found an IFN-dependent increase in plasma membrane rigidity<sup>801</sup>. IFN increases membrane rigidity within 30 minutes and maintains it for two days, making it a potent antiviral strategy for preventing viral infection and transmission<sup>802</sup>. Likewise, 25-HC limits the development of several enveloped viruses, including HIV, by preventing virus-host cell membrane fusion<sup>803</sup>. On the other hand, type I IFNs cause an increase in unsaturated fatty acids and a decrease in saturated fatty acids in membrane contents<sup>801</sup>. Treatment with IFN- $\alpha$ 2 lowers the viscoelastic properties and membrane fluidity of red blood cells in individuals with chronic hepatitis C infection, which may result in hemolytic anemia, a common adverse effect of IFN therapy<sup>804</sup>. Furthermore, type I IFNs reduce polyamine levels, which has a powerful antiviral impact and suppresses RNA virus replication. Polyamines are required for the transcription and translation of viral RNA and proteins<sup>805</sup>.

IDO1 is an intracellular, non-secreted enzyme that catalyzes the synthesis of kynurenine derivatives from the essential amino acid, tryptophan. Two IFN-stimulated response elements and three IFN-activated sites were identified in the IDO1 promoter region. As a result, IFN expression can be increased. IFN-induced IDO1 activation lowers serum tryptophan levels. As many microbial species rely on this amino acid, its breakdown by IDO1-expressing innate immune cells appears to be an important immunological response against bacterial

infections<sup>806</sup>. IFN-induced IDO1 activation lowers serum tryptophan levels. Because many microbial species rely on this amino acid, its breakdown by IDO1-expressing innate immune cells appears to be an important immunological response against bacterial infections<sup>807</sup>. In viral infections, however, IFN-induced IDO1 activation appears to be typically detrimental due to the formation of an immunotolerogenic milieu. T cell activation and proliferation require tryptophan. T cell activation and proliferation require tryptophan. Tryptophan deficiency suppresses T cell immunity, and kynurenine derivative build-up promotes T cell tolerance by inducing T<sub>REG</sub> cells<sup>806</sup>. Finally, the induction of IDO and depletion of amino acids, such as leucine, arginine, and tryptophan, decreases mTORC1 activity<sup>808</sup>.



# OBJECTIVES

CD4+ T cells are the primary targets of HIV. However, these lymphocytes do not represent a homogeneous population. Indeed, T cell differentiation is coupled with substantial alterations in a variety of cellular processes, allowing for the adaptations required to meet the energetic requirements for effector functions and cell expansion potential. These adaptations generally entail alterations in cellular metabolism, which act as both a consequence and a trigger for cellular differentiation<sup>266</sup>. When previously quiescent T cells receive differentiation signals, they must adapt to cellular functions and balance catabolic and anabolic demands rather than undertake mostly catabolic reactions. When stimulated, CD4+ T cells adapt by increasing metabolite uptake, transfer, and utilization to support major needs faced by rapidly proliferating immune cells: energy production and biosynthetic precursor synthesis.

Interestingly, several studies have shown a link between metabolism and HIV infection in CD4+ T cells<sup>362,363,368,375,809</sup>. Furthermore, our team has shown that differences in HIV permissibility between naïve and more differentiated CD4+ T cell subsets are associated with the metabolic activity of these cells<sup>361</sup>. We found that HIV preferentially infects cells with relatively high metabolic activity, and the hierarchy of metabolic activities (OXPHOS and glycolysis) of CD4+ T cell subsets matched the hierarchy of infection of the same subsets ( $T_{EM} > T_{TM} \geq T_{CM} > T_N$ ). This was further reinforced by the finding that HIV-infected cells had higher metabolic activity than cells that had been exposed to the virus but remained uninfected. Moreover, suboptimal inhibition of glycolysis blocked the early post-entry steps of viral replication and HIV reactivation in CD4+ T cells from ART-treated individuals. The buildup of biomass that accompanies the increased cellular metabolism in CD4+ T cells may provide the virus with an abundance of factors that are necessary for their replication.

Immunometabolism, the study of the interplay between bioenergetic pathways and immune cell functions, is a new concept in immunology that is gaining recognition as an emerging field in cancer research. The role of cellular metabolism in infectious diseases, notably HIV, is just being appreciated and the relative relevance of metabolic pathways in HIV infection is poorly described.

Consequently, the primary purpose of my dissertation research was to address the following questions:

> **What is the relative contribution of cellular metabolism to replication-competent HIV infection in CD4+ T cells?**

Findings from our lab imply that infection of primary CD4+ T cells relies on glycolysis. The application of 2-DG, a glucose competitive inhibitor known to inhibit cancer cell proliferation and tumor growth, inhibited HIV replication and induced the specific death of infected cells. This and other studies suggested that glutamine metabolism may also be necessary for optimal infection of CD4+ T-cells. However, these studies were mostly conducted using single-cycle VSV-G pseudotyped viral particles lacking HIV env and nef.

CD4 + T cells overexpress various transporters to fuel their increased biosynthetic demands. They also increase flux through metabolic pathways that produce precursors, such as the pentose phosphate pathway (PPP), to produce nucleotide precursors and NADPH to combat oxidative stress and glutaminolysis to replenish the tricarboxylic acid (TCA) cycle and produce NADPH. This leads to the increased consumption of many nutrients by CD4+ T cells, including glucose, lactate, pyruvate, glutamine, and fatty acids. The relative contributions of many metabolic pathways involving these nutrients, such as OXPHOS, glutaminolysis, FAO, and FAS, remain unclear. These different metabolic pathways generate diverse byproducts that might be complementarily exploited by HIV to complete different steps of its replication cycle.

Deregulation of cell energy metabolism is considered a hallmark of cancer cells, which holds great promise for new therapeutic interventions. This has boosted the development of multiple metabolic inhibitors, some of which are already being tested in clinical trials, and can be used to decipher the implications of the stages of the different metabolic pathways in cellular function. We employed inhibitors that target different pathways of cellular metabolism to evaluate how unbalanced metabolic fluxes impact HIV infection with a replication competent virus and the survival of infected cells. We studied CD4+ T cells from HIV-negative donor blood that were activated using a “suboptimal activation” strategy in order to induce as few changes as possible so that the intrinsic metabolic features of the cultured cells remain as closely as possible related to that of peripheral CD4+ T cells under physiological conditions.

> **How some cytokines, known to be released during acute infection, alter cell metabolism and influence HIV-1 infection?**

The contribution of CD4+ T cell subsets to HIV reservoirs has been reported to differ during acute and chronic infection, the reservoir is more labile during primary infection, and some long-lived populations (such as central memory cells) are thought to be more resistant to infection in this period. The differences in reservoir composition between the primary and the chronic phases might be due to the impact of the extracellular milieu, which is known to be pro-inflammatory during the acute phase and generally characterized by the presence of a “cytokine storm”, a common feature of acute infections. Several cytokines that are secreted during the immune response against HIV are known to have an impact on cellular metabolism and the permissibility of cells to HIV, as discussed in the introduction. Moreover, in a previous study of the team, strong positive correlations were found between the expression of genes related to metabolic pathways and the expression of genes related to the IFN response (unpublished). We then hypothesized that cytokines released during acute infection influence the establishment of the HIV reservoir by altering the metabolic activity of the cells.

Cytokines can affect cellular activation, differentiation and effector function.  $\gamma$ c cytokines, in particular, are known to play an important role in dictating the type of cellular response that is developed. Cytokines can induce a specific functional phenotype depending on the target cell and the availability or absence of other soluble factors in the milieu. Cytokines dictate the fate of immune cells by recruiting different metabolic pathways in the cell to support specific source of energy, synthesis of specifically needed intermediates/building blocks. We therefore conducted a comparative study of the impact of a panel of relevant cytokines on primary CD4+ T cell activation, differentiation, metabolic activity and susceptibility to HIV-1 infection. We

studied  $\gamma$ c cytokines, for the role they play in T cell homeostasis and function. We also chose IL-10 for its counteracting effects against pro-inflammatory cytokines additionally it has been suggested to play a role in HIV infection, and IFN- $\alpha$  for its antiviral capacities. We finally used the CD4 ligand IL-16 as a reference control.

The overall goal of this thesis was to provide new insights into how the modulation of cell metabolism affects the establishment of HIV-1 infection in CD4+ T cells, and pave the way for the implementation of new therapeutic interventions against HIV-1 using small molecules and/or cytokines targeting immunometabolism.

**CHAPTER I. HIV-1 INFECTION OF CD4+ T  
CELLS IS DEPENDENT ON VARIOUS  
METABOLIC PATHWAYS THAT CAN BE  
TARGETED USING SPECIFIC INHIBITORS**

## **HIV-1 infection of CD4+ T cells is dependent on various metabolic pathways that can be targeted using specific inhibitors**

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### **Keywords**

HIV-1, CD4+ T cells, Immunometabolism, Metabolism, Glycolysis, Glutaminolysis, OXPHOS, FAS, FAO

## **ABSTRACT**

Despite its success, antiretroviral therapy does not cure HIV-1 infection because of the presence of reservoirs of persistently infected cells harboring a replication-competent HIV-1 genome. The main cellular reservoir for these HIV-1 proviruses is CD4<sup>+</sup> T cells. Previous studies have shown that cellular metabolism is a key determinant of reservoir seeding in these cells, and its inhibition represents an approach to tackling HIV-1 infection and potentially eliminating HIV-infected CD4<sup>+</sup> T cells. To better understand the metabolic pathways favoring HIV-1 infection in CD4<sup>+</sup> T cells, we tested different compounds targeting a broad range of metabolic pathways. We found that inhibition of glycolysis, pyruvate import through mitochondrial pyruvate carriers (MPCs) and fatty acid synthesis consistently decreased the frequency of HIV-infected CD4<sup>+</sup> T cells. We also observed a higher sensitivity to metabolic inhibition in infected cells compared to non-infected cells. In contrast, the inhibition of glutaminolysis and fatty acid  $\beta$ -oxidation, although reducing T cell activation, did not reduce HIV-1 infection. These results show that different metabolic pathways are important for HIV-1 infection of CD4<sup>+</sup> T cells, and that their effects are at least partially independent of changes in T cell activation.

## **INTRODUCTION**

Current antiretroviral therapy (ART) can successfully limit HIV-1 replication and prevent or reverse immunodeficiency in people with HIV (PWH). However, virus replication typically resumes within weeks following treatment cessation. A limited pool of CD4<sup>+</sup> T cells harboring replication competent HIV-1 proviruses is the main obstacle to a cure. It is thought that this reservoir is established very early during the primary phase of HIV-1 infection and that early ART initiation shortens the seeding of this reservoir, offering better clinical outcomes for PWH (Massanella et al. 2021). Identifying the factors behind the seeding and survival of HIV-infected cells is a priority in the quest for an HIV cure. Upon activation, T cells undergo clonal expansion, and it is generally believed that the intracellular milieu associated with cell cycle entrance is favorable for HIV-1 infection. Although cytokines, chemokines, and antigenic signals have been extensively studied in relation to their role in promoting HIV-1 infection, more recent research suggests that cellular metabolism influences T-cell function as well as permissibility to infection. Other viruses have been described to possess different mechanisms to enhance cell metabolism to favor their replication (Goodwin, Xu, and Munger 2015; Sanchez and Lagunoff 2015). Several retroviruses, for instance, have evolved to exploit metabolite transporters as cellular receptors. For example, GLUT1 is the primary receptor for HTLV-1 (Manel et al. 2003); the phosphate transporters PiT1 and PiT2 have been identified as surface receptors for koala retrovirus, feline leukemia virus, and murine leukemia virus (von Laer et al. 1998; Oliveira, Farrell, and Eiden 2006; Y et al. 1992); and the amino acid transporters ASCT1 and ASCT2 are the receptors for the feline RD-114 endogenous retrovirus (Shimode et al. 2013). HIV-1 does not employ metabolite transporters as cell entry receptors; nevertheless, glucose transporter 1 (GLUT1) expression has been shown to be essential for early HIV-1 replication steps in CD4<sup>+</sup> T cells. (Loisel-Meyer et al. 2012). Uptake of GLUT1 was shown to regulate the permissibility of CD4<sup>+</sup> T cells to HIV-1 infection (Loisel-Meyer et

al. 2012) and HIV-1 preferentially infected *in vitro* CD4<sup>+</sup> T cells expressing GLUT1 and OX40 (Palmer et al. 2017). Results from our team showed that HIV-1 preferentially infects CD4<sup>+</sup> T cells that exhibit high glycolytic capacity and oxidative phosphorylation and that suboptimal inhibition of glucose uptake and glycolysis blocked single-cycle VSV-G pseudotyped HIV-1 infection, selectively eliminated recently infected CD4<sup>+</sup> T cells and impaired HIV-1 amplification from reservoir cells from PWH. Additionally, HIV-1 infection has been found to be associated with increased intracellular glutamine levels (Hegedus et al. 2017). Hence, cellular metabolism appears to also play a critical role in HIV-1 infection of CD4<sup>+</sup> T cells and deserves additional exploration, as it may be a potential target for new therapies against HIV-1. While glycolysis have been hypothesized to be important for early steps of HIV-1 infection, potentially for its capacity to fuel the pentose phosphate pathway which results in the production of deoxynucleotides required for reverse transcription, the importance of glutamine metabolism infection have been attributed to the contribution of  $\alpha$ -ketoglutarate ( $\alpha$ -KG), a glutamine intermediate, to the TCA cycle and OXPHOS, which were also found to augment HIV-1 reverse transcription. Acetyl-CoA, which is generated from glycolysis and pyruvate, can also enter the TCA cycle and generate  $\alpha$ -KG.  $\alpha$ -KG activates the mammalian target of rapamycin (mTOR), a key regulator of cellular metabolism and diverse parallel pathways (Waickman and Powell 2012), implicated in HIV-1 latency in CD4<sup>+</sup> T cells (Besnard et al. 2016). Other metabolic pathways appear to interfere with later steps of HIV-1 replication, such as viral transcription, assembly or budding and the survival of infected cells. Deregulation of cellular energetic metabolism is an emergent characteristic of cancer that points at promising therapeutic targets for cancer therapy. This is promoting the development of diverse metabolic compounds, some of which are already in clinical trials, which can be used to decorticate the implication of the stages of distinct metabolic pathway in cellular function (Y. Zhao, Butler, and Tan 2013) and



may offer new opportunities for the treatment of HIV-1 infection. We explored here the impact of different families of metabolic inhibitors on HIV-1 infection of CD4+ T cells.

## **RESULTS**

### **Impact of metabolic inhibitors on cellular activation**

We first assessed the impact of metabolic drugs on cellular activation levels by evaluating the expression of common T lymphocyte cell surface activation-associated molecules, CD69, CD25, and HLA-DR. CD4+ T cells from HIV-negative blood donors were cultured for five days in the presence of soluble anti-CD3, washed, and cultured in the presence of different metabolic molecules for three days before running the activation panel. This "suboptimal" activation strategy previously allowed us to preserve the inherent differentiation and HIV-1 susceptibility of CD4+ T cells (Valle-Casuso et al. 2019) and uncover disparities in HIV-1 susceptibility of CD4+ T cells from different individuals (Sáez-Ciri3n et al. 2011). The use of 2-Deoxyglucose (2-DG) at 5 mM, a glucose analog that blocks glycolysis by competing with glucose to bind hexokinase (the first rate-limiting enzyme of glycolysis), resulted in the downregulation of all three activation markers analyzed (Figure 1A). Zaprinast (4 $\mu$ M), a well established inhibitor of cGMP-specific phosphodiesterase (PDE), specifically inhibits mitochondrial pyruvate carriers (MPC) and potently blocks mitochondrial pyruvate transport (Du et al. 2013), also downregulated the expression of all three activation markers (Figure 1B). The glutamine analog, 6-Diazo-5-oxo-1-norleucine (L-DON) at 50 mM also resulted in a significant decrease in CD25 and CD69 (Figure 1C). The FDA-approved anti-obesity drug orlistat at 10  $\mu$ M, a fatty acid synthesis inhibitor known to elicit its effects by inhibiting the thioesterase domain of fatty acid synthase (FASN), showed a significant decrease in CD25 and HLA-DR (Figure 1D). Therefore, although the metabolic inhibitors that we used overall

decreased the activation status of the cells, they did not equally impact the different activation markers analyzed.

### **Suboptimal inhibition of different metabolic pathways hinders HIV infection levels**

Previous results from our lab showed that inhibition of cellular metabolism can be used to target HIV infection. However, most of the previous experiments were conducted using single-round VSV-G pseudotyped HIV particles. We therefore investigated more largely if blocking metabolic pathways decreased the level of replication-competent HIV BaL strain in primary CD4<sup>+</sup> T cells. We confirmed our previous results showing that the presence of 5 mM of 2-DG reduced productive HIV-1 infection (Figure 2A). To better understand the implication of glucose metabolism on HIV infection, we used 3-bromopyruvate (3-BrPA), which also dampens the glycolytic rate but acts differently than the glucose analog 2-DG and directly targets the glycolytic enzyme hexokinase-II (HK-II). 3-BrPA at 5  $\mu$ M also decreased HIV-1 p24 levels in most of our samples (Figure 2B). We also investigated if the decrease in infection rates caused by 2-DG was associated solely with the impairment of the glycolytic pathway or was a result of shortage in pyruvate, which is a critical metabolite that links glycolysis with the mitochondrial tricarboxylic acid (TCA) cycle, a hub for the synthesis of amino acids, carbohydrates, and fatty acids. Zaprinast (at 4  $\mu$ M) resulted in a significant decrease of p24 protein levels in CD4<sup>+</sup> T cells (Figure 2C) which suggests that oxidative phosphorylation is also important to HIV-1 infection.

Next, we proceeded to study the capacity of inhibitors of glutamine metabolism, which has been shown to play an important role in the early steps of the HIV-1 replication cycle (Clerc et al. 2019), to block productive HIV-1 infection. We used the glutamine analog L-DON, which we had previously shown to be able to block single cycle infection with VSV-G pseudotyped HIV-1 particles. Surprisingly, we did not observe a significant impact of DON on the levels

p24-positive CD4<sup>+</sup> T cells infected with HIV-1 BaL. We did not find any noticeable effect either when using an allosteric inhibitor of glutaminase 1 (GLS1), bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES) (Hartwick and Curthoys 2012; Katt et al. 2012) (Figure 2D and 2E).

We next investigated whether inhibition of fatty acid metabolism could influence HIV-1 infection. Clinically approved compound orlistat, which targets fatty acid synthase (FASN), at 10  $\mu$ M resulted in a decrease in the proportion of p24 positive CD4<sup>+</sup> T cells (Figure 1F). To further assess the effect of targeting the fatty acid synthesis pathway, we used 5-(tetradecyloxy)-2-furoic acid (TOFA), an allosteric inhibitor of acetyl CoA carboxylase (ACC), and C75, a mycotoxin and FASN inhibitor that is directly downstream of ACC. Both TOFA and C75 at 20  $\mu$ M and 50  $\mu$ M respectively, decreased HIV-1 infection of CD4<sup>+</sup> T cells (Figures 1G and 1H). In contrast, Etomoxir (ETO), a known Carnitine palmitoyl transferase I (CPT-1) and fatty acid oxidation inhibitor did not reduce (at 10  $\mu$ M) the frequency of p24-positive cells but rather tended to increase the frequency of infected cells (Figure 1I).

Finally, Metformin (MET), a biguanide oral anti-hyperglycemic drug used in the treatment of patients with non-insulin-dependent diabetes, which was found to inhibit complex I (NADH:ubiquinone oxidoreductase) of the electron transport chain (ETC), thus blocking the respiratory chain and ATP production in the mitochondria, appeared to promote HIV-1 infection in most samples tested (Figure 1J).

Overall, these results show that small molecules targeting glucose metabolism and fatty acid synthesis consistently impaired HIV-1 infection of CD4<sup>+</sup> T cells, whereas molecules targeting glutamine metabolism and fatty acid oxidation did not efficiently block infection under our experimental conditions.

### **Selective effect of metabolic modulators on highly metabolic CD4<sup>+</sup> T cell subsets**

We observed that the treatment of CD4<sup>+</sup> T cells with different metabolic modulators affected the surface expression levels of CD4 and CD3 (Figure 2A, 3A). We found that cells expressing high levels of CD3 and CD4 were highly metabolic and characterized by higher expression of GLUT1 and higher uptake of the glucose fluorescent analog 2-NBDG (Figure 3A and 3B), which is in agreement with our previous observations (Valle-Casuso et al. 2019). Notably, we noticed that this specific population of CD3<sup>hi</sup>CD4<sup>hi</sup> cells disappeared when cells were treated with metabolic modulators (Figure 3A), representing a good proxy for assessing the efficacy of the molecules tested in metabolic cellular processes. We found that the use of different metabolic modulators affected the establishment of this CD3<sup>hi</sup>CD4<sup>hi</sup> population independently of the metabolic pathway they targeted (Figure 3C).

### **Activation status does not always correlate with infection rate**

Our results indicated that the metabolic modulators that we employed here were able to interfere with cellular processes such as CD4<sup>+</sup> T cell activation, but this did not systematically have an impact on HIV infection. Moreover, we did not observe a correlation between the downregulation of CD25, CD69 and HLA-DR expression and decreased HIV-1 infection following the use of metabolic drugs targeting glycolysis, glutaminolysis, or fatty acid synthesis (Figure 4A, 4B and 4C). Accordingly, drug-induced downregulation of the CD3<sup>hi</sup>CD4<sup>hi</sup> population was not always accompanied by a decrease in HIV-1 infection (Figure 4D). These results further confirm our previous observations that, although T cell activation and T cell metabolism are tightly intertwined, cell metabolism can alter CD4<sup>+</sup> T cell susceptibility to infection independently of the activation status of the cells.

### **Metabolic drugs show higher toxicity in infected compared to non-infected CD4+ T cells**

Finally, we wondered whether HIV-infection could enhance the sensitivity of cells to metabolic inhibitors. We observed higher drug-induced toxicity with increasing concentrations of metabolic modulators in infected CD4+ T cells when compared to non-infected cells (Figure 5). This effect was less noticeable for some drugs, such as 2-DG and L-DON, where only a 10% difference in viability between the non-infected and infected cells was observed (Figure 5A and 5B). In contrast, a sharp difference in viability was observed for cells cultured in the presence of 50  $\mu$ M orlistat, a concentration that did not affect the viability of non-infected cells (96% viability) but strongly compromised the viability of HIV-1 BaL-exposed cells (29% viability) (Figure 5C). Similarly, only 12% of HIV-1 exposed cells were viable after challenge with 3-BrPA at 30  $\mu$ M, while the viability of non-infected CD4+ T cells was 65%. Overall, our results indicate that HIV-1 infection can be blocked using metabolic modulators and that HIV-1 infection itself provokes changes in the metabolic activities of the cells, rendering them more susceptible to the cytotoxic effects of these drugs.

## **DISCUSSION**

Over the last few years, our team has explored the role of cellular metabolism in establishing HIV-1 infection and reservoir seeding. Our previous results suggested that targeting the cellular metabolism of CD4+ T cells might be a promising strategy to tackle infection and the persistent HIV-1 reservoir in PWH. Here, we extended our observations by exploring the impact on productive HIV-1 infection of several compounds that modulate metabolic pathways. Our results point to the prominent effect of drugs targeting glucose metabolism and fatty acid synthesis as potential tools to tackle HIV-1 infection.

We observed a significant decrease in p24-positive cells in the presence of 2-DG and 3-BrPA, suggesting that impairment of the glycolytic pathway by these two molecules alters HIV-1 replication. The inhibition of pyruvate, a product of glycolysis, transport into the mitochondria for oxidative respiration, and TCA cycle fueling by Zaprinast also results in a significant decrease in HIV-1 infection. Fatty acid synthesis was also found to be important for HIV-1 replication in CD4<sup>+</sup> T cells, as the use of TOFA, C75 and Orlistat resulted in a decrease in the frequency of infected cells. Both C75 and orlistat target FASN, whereas TOFA targets ACC. However, C75 and orlistat do not inhibit the same subunits of FASN. Orlistat inhibits the thioesterase domain, which catalyzes the release of the end product palmitate. C75 targets the  $\beta$ -ketoacyl synthase domain of the same enzyme. C75 is known to induce higher apoptosis in human cancer cells than orlistat by causing an increase in malonyl-CoA intracellular levels (which is cytotoxic; the accumulation of malonyl-CoA increases the cellular level of ceramide, contributing to apoptosis induction). Paradoxically, C75 also activates fatty acid  $\beta$ -oxidation, which is usually inhibited by high malonyl-CoA levels (Thupari et al. 2002). Fatty acid synthesis, regulated by FASN, and fatty acid oxidation, regulated by CPT-I and CPT-II, are key anabolic and catabolic pathways linked by their common metabolic intermediate, malonyl-CoA. C75 induces  $\beta$ -oxidation by acting on the malonyl-CoA-insensitive component of the carnitine palmitoyltransferase system CPT-II (Nicot et al. 2004). While TOFA and Orlistat may have impacted HIV-1 infection by reducing endogenous fatty acids that contribute to phospholipid composition of the cell membrane, C75 adds to the inhibition of fatty acid synthesis, the cellular toxicity caused by the accumulation of malonyl-CoA levels, as well as an increase in  $\beta$ -oxidation (Puig et al. 2008). Although C75 inhibited HIV-1 infection, infection levels were not impaired by ETO, a molecule that inhibits fatty acid oxidation by blocking CPT-I, an enzyme associated with the outer mitochondrial membrane that transfers a long-chain acyl group from coenzyme A to carnitine, a transformation that allows for the transport of long-

chain fatty acids to the mitochondrial matrix. Although ETO has been reported to inhibit the mitochondrial adenine nucleotide transporter, as well as complex I of the ETC (Divakaruni et al. 2016), this off-target effect of the CPT inhibitor is seen when the used concentration of ETO exceeds 10  $\mu$ M, which is the maximum concentration used in this study.

We were surprised to see that inhibition of glutamine-utilizing enzymes by L-DON and, GLS1 specifically, by BPTES did not result in impairment of HIV-1 infection. It has been shown that HIV-1 infected macrophages have an increased production of glutamate, which is produced from glutamine by glutaminases and glutamine amidotransferase activities, and that L-DON reduces this conversion (Jianxing Zhao et al. 2004). Furthermore, study by Clerc et al. have shown the dependency of HIV-1 early replication steps on glutaminolysis and that exogenous  $\alpha$ -KG rescues HIV-1 infection by promoting reverse transcription (Clerc et al. 2019). However, the later study emphasized the importance of glutamine in fueling the TCA cycle through the conversion of glutamate to  $\alpha$ -KG, which is used in the citric acid cycle. Although, L-DON competes with glutamine uptake and glutamine utilizing-enzymes while BPTES impairs glutaminolysis through uncompetitive inhibition of GLS1, other pathways could continue fueling the TCA cycle when glutamine is lacking and glutaminolysis is inhibited. For instance, glycolysis, through the generation of pyruvate, fuels the TCA cycle. When glutamate is lacking, the cell may adapt and compensate by increasing their glycolytic rate. This mechanism of compensation has been observed with MPC inhibitors. The same phenomena could be observed when glutamine is scarce but glucose is abundant, which is a characteristic of most *in vitro* culture media. This type of metabolic reprogramming to utilize TCA cycle inputs different from glutamine has already been described in the context of cancer (Lukey et al. 2019; Dos Reis et al. 2019; Singleton et al. 2020). The same compensation mechanism could exist also when we blocked glycolysis using 2-DG and 3-BrPA. However, besides blocking glycolysis and

intermediates necessary to fuel the TCA cycle, 2-DG and 3-BrPA block the pentose phosphate pathway which among other functions, provides the cell with nucleosides necessary for the cell as well as for HIV-1 reverse transcription activity. Although L-DON has also been shown to inhibit multiple glutamine amidotransferase involved in *de novo* purine and pyrimidine synthesis, its lack of large spectrum of targets necessitates very high doses for it to efficiently block nucleosides synthesis (Hartman~ 1963). Of note, L-DON has a weak millimolar inhibitory potency and in our experiments, we limited its concentration to a micromolar level because of its high toxicity in primary CD4+ T cells (Thomas et al. 2013). We observed however an enhanced cytotoxicity of L-DON on infected cells vs non-infected cells at the higher doses tested, which suggest that infection may indeed mobilize the glutamine pathway making them more sensitive to its inhibition.

Surprisingly, MET, an inhibitor of the complex I of the ETC and oxidative phosphorylation, did not impair HIV-1 infection, but rather showed an opposite enhancing effect. It is important to mention that one of the downsides with compounds that alter cellular metabolism is the lack of extreme specificity and the existence of off-target effects. Despite vast clinical experience, the mechanism of action of MET is still not fully understood. In comparison with other drugs, MET is given at large doses, in the range of 2 g per day, suggesting that the effect may not be a result of the drug's interaction with a specific protein target, but rather due to multiple mechanisms (Rena, Hardie, and Pearson 2017). MET has been found to be an upstream inhibitor of mTOR, functioning through activation of AMPK sensing pathway and thus modulating glycolysis in T cells. Modest decreases in ATP production can result in relative increases in AMP or ADP. An elevated AMP/ATP ratio triggers the activation of AMPK, the main bioenergetic sensor in eukaryotic cells, which promotes catabolic reactions that generate more ATP and inhibit energy-consuming anabolic pathways. However, conflicting studies exist



regarding how MET inhibits mTOR and specifically mTOR complex I (mTORC1) signaling. According to several studies, MET reduces ATP generation by suppressing the mitochondrial respiratory chain complex I and decreasing oxidative phosphorylation levels in cells, which results in the activation of AMPK and the subsequent inhibition of the mTOR/mTORC1 signaling pathway (Dowling et al. 2007; Howell et al. 2017; Morales and Morris 2015). Other studies state that MET-induced mTOR regulation is entirely independent of AMPK (Kalender et al. 2010; Isaam Ben Sahra et al. 2011). MET has also been shown to induce a compensatory increase in the cellular glycolytic capacity to supply sufficient energy to the cells in the absence of oxidative phosphorylation (Alhourani et al. 2021; Jing Zhao et al. 2019). Interestingly, a Cohort study of PWH with diabetes revealed the association of MET treatment with improved CD4<sup>+</sup> T cell recovery (Moyo et al. 2014). MET is proved to normalize mitochondrial dysfunction of CD4<sup>+</sup> T cells, delay diseases of aging such as heart disease, cancer and Alzheimer, and alleviate aging-related inflammation (Bharath et al. 2020). Therefore, MET is believed to elicit its effects of CD4<sup>+</sup> T cell recovery through normalizing mitochondrial function and regulating glycolysis, which provides anti-inflammatory effect and could help restore CD8<sup>+</sup> T cell function, and not by having a direct impact on viral replication (Routy et al. 2019). Finally, MET have been found to interfere with NF- $\kappa$ B and STAT3 signaling which play an important role in antiviral immune responses and cytokine signaling (Moiseeva et al. 2013; Vasamsetti et al. 2015).

HIV-1 infection has been commonly associated to the cellular activation of CD4<sup>+</sup> T cells. A high activation status is almost always linked to a high metabolic activity, however we show that the effects elicited by the metabolic modulators used in this study are not solely caused by alteration of cellular activation of the cells. We describe in our experiments a particular population characterized by the highest expression of CD3 and CD4 surface markers

(CD3<sup>hi</sup>CD4<sup>hi</sup>). We demonstrated that the CD3<sup>hi</sup>CD4<sup>hi</sup> population corresponds to highly metabolic cells, and it shows higher expression levels of GLUT1 and higher uptake of 2NBDG compared to the rest of CD4<sup>+</sup> T cells. We found that the use of different metabolic drugs, targeting different metabolic pathways including glycolysis, provoked a decrease of the CD3<sup>hi</sup>CD4<sup>hi</sup> population. Although we are aware that we did not directly test the effects of these drugs on cellular metabolism, we hypothesized that the effects on the distribution of CD3<sup>hi</sup>CD4<sup>hi</sup> population are representative of metabolic changes in the cells caused by the utilized metabolic drugs.

Increasing evidence point towards the importance of cellular metabolism in viral infections. Examples vary from viruses that use specific nutrient transporters to enter a host cell, viruses that hijack and reprogram the host cell metabolism for their benefit and viral infections that are blocked when cellular metabolism is impaired. In HIV-1 infection, active glycolysis is required for viral particle fusion. Glycolysis activity affects HIV-1 fusion by modifying the order and tension of the T cell membrane (Coomer et al. 2020). Viral entrance and egress are two membrane-regulated activities in human cells that are significantly influenced by membrane order and tension (Ewers and Helenius 2011; Kozlov and Chernomordik 2015). Additionally, abundance in acetyl-CoA leads to the acetylation and stabilization of microtubules, which facilitates cytoplasmic transport of viral products and promote entry and egress from the nucleus in an infected cell (Taylor et al. 2020). Acetyl-CoA is also a metabolite used by histone acetyltransferases (HATs) to transfer an acetyl group onto a histone lysine. Thus, acetyl-CoA can also aid HIV transcription (Lint et al. 1996). Both glycolysis and glutaminolysis are key metabolic pathways that have been proposed to be mobilized to sustain the intracellular steps of viral replication, and fatty acid synthesis is important for the formation of the viral envelope and virus-containing compartments and the production of new viral particles (Sanchez and

Lagunoff 2015). In despite of all these findings, metabolic drug targeting is not yet utilized and remains a poorly understood area in HIV-1 and other viral infections.

Most of the molecules tested here were chosen for having been clinically approved, being tested in clinical trials, or having been used in preclinical studies in animal models. Compounds like MET and orlistat are drugs that are used in metabolism-related disorders such as diabetes and obesity. Zaprinast, a lead compound used for the development of sildenafil (Viagra), is known as a moderate inhibitor for cGMP-PDEs, especially PDE5 and PDE6 that independently of its known effects on PDE, has been shown to potently inhibit pyruvate transport into the mitochondria. 2-DG was used in phase I and II clinical trials but stopped at phase III due to its limited anticancer properties. 2-DG is however considered to be safe and “well” tolerated. L-DON was the most extensively used inhibitor until the last decade but its clinical development was abandoned after phase I and II trials of L-DON given intermittently at high doses were hampered by excessive toxicity compared to the little therapeutic potential (Pinkus 1977). Recently however, the recognition that multiple tumor types are glutamine dependent has renewed interest in glutaminolysis inhibitors such as L-DON. This interest lead to the development of more specific compounds such BPTES, which is currently one the most studied glutaminase inhibitor (Robinson et al. 2007). A BPTES analog, CB-839, has advanced to clinical trials for some types of cancers (Lee et al. 2020; Meric-Bernstam et al. 2016; Milano et al. 2022; Study of the Glutaminase Inhibitor CB-839 in Solid Tumors n.d.). Finally, the use of 3-BrPA, ETO, C75 and TOFA, which we used to explore more largely the effect of the previous molecules on particular metabolic pathways, have so far been limited to preclinical studies.

In cancer, the use of metabolic drugs that have a broad action that impacts both healthy and cancerous cells at optimal doses, is proven non-toxic for healthy cells because the non-

physiological metabolic state caused by the drugs, can be well tolerated by normal cells, while proving to be cytotoxic to many types of cancer cells. This observation can also be true for non-infected versus HIV-1 infected cells. We noticed that drug toxicity is increased when the same drug at the same concentration is used in HIV-1 exposed cells in comparison to non-exposed cells. This observation justifies further the use of metabolic modulators and immunometabolism as a new targets towards HIV-1 management, reservoir elimination and cure.

In the present work, we did not test for different combinations of metabolic drugs. Combining two or more compounds can prove very efficient, by solving the problem of compensating parallel pathways. Because cancer cells have high glycolytic activity, a big amount of glucose is consumed and a high level of ATP is synthesized, resulting in a drop in the AMP/ATP ratio and drastically reduced AMPK activity (Rehman et al. 2014). As a result, in recent years, both 2-DG and MET, which activate AMPK, have been employed in the treatment of malignancies. The combination of MET and 2-DG can greatly deplete cancer cells' ATP supply and block the over-activation of proliferative signaling pathways in cells, hence dramatically suppressing tumor cell over-proliferation (Issam Ben Sahra, Tanti, and Bost 2010; Xue et al. 2016). The use of combinations of two or more drugs also reduces the side effects caused by high doses of the individual drugs (Bikas et al. 2015). To further limit the cytotoxic effects of such drugs, precise interventions to target cell subsets are being studied so as to keep other regular cell functions undisturbed. Drug delivering system in oncology might provide some clues, such as active-ingredient-loaded nanoparticle directed by specific cell recognizing ligand coating. An example is a latency reverse agent-loaded anti-CD4 liposomes that was selectively uptaken by CD4<sup>+</sup> T cells (Jaimalai et al. 2020).

Our work, although limited, offers a better grasp of the role that cellular metabolism plays in HIV-1 infection and serves as an inspiration for further studies in this area. HIV-1 infection is a complex system controlled by parallel pathways that include cellular metabolism, with multiple accelerators and different breaks that could be targeted in combination in order to efficiently counteract HIV-1 pathogenesis. The continuation of this work could help us to identify combination of metabolic molecules that could be explored in proof of principle pre-clinical trials in non-human primate models of SIV infection, to test their efficiency at blocking infection, eliminating infected reservoir cells and alleviating HIV-1 infection.

## **MATERIAL AND METHODS**

### **Isolation and culture of primary human CD4<sup>+</sup> T cells**

Primary human cells were used in all of the experiments. As part of an agreement with the Institut Pasteur (C CPSL UNT, number 15/EFS/023), blood samples from volunteer donors were procured from the French blood bank (Établissement Français du Sang).

Peripheral blood mononuclear cells (PBMCs) were isolated from donor-derived buffy coats. Buffy coats were diluted 1:1 (vol/vol) in a phosphate-buffered saline (PBS) buffer, overlaid on a density gradient medium, Lymphocyte Separation Medium (EuroBio) at a ratio of 2:1 blood to Ficoll and then centrifuged at 1,800 rpm for 30 min at minimum acceleration/deceleration to harvest PBMCs. Red blood cells (RBC) were removed using 1X RBC lysis buffer.

CD4<sup>+</sup> T cells were then purified from the PBMCs by negative selection using Stemcell EasySep Human CD4<sup>+</sup> T cell enrichment kit. CD4<sup>+</sup> T cells-enriched PBMCs show purities greater than 90%. Cells were cultured at a density of 10<sup>6</sup> cells/ml in RPMI 1640 containing Glutamax (ThermoFisher), supplemented with 10% fetal calf serum (FCS), penicillin (10 IU/mL) and streptomycin (10 µg/mL) (referred to below as culture medium). Cells are then maintained at 37°C in a standard tissue culture humidified incubator containing atmospheric (20%) O<sub>2</sub> and 5% CO<sub>2</sub>.

### **Suboptimal activation with anti-CD3 and IL-2**

Depending on the condition, CD4<sup>+</sup> T enriched PBMCs were stimulated by adding a soluble anti-CD3 monoclonal antibody (0.5 µg/mL; clone UCHT-1; Biolegend) and IL-2 (Miltenyi; 100 U/mL) to the culture medium (activated condition, ACT). Cells were then maintained in culture for 5 days prior to infection or analysis as previously described (Valle-Casuso et al. 2019).

### **Infection of primary CD4<sup>+</sup> T cells**

Activated CD4<sup>+</sup> T cells were infected with HIV-1 BaL (R5) virus (0.3 ng of p24 per 10<sup>6</sup> cells) by spinoculation for 1 hour at 1,200 × g at room temperature and then incubated for 1 h at 37°C in a humidified 5% CO<sub>2</sub> incubator (O'Doherty, Swiggard, and Malim 2000). Cells were then washed once with PBS and incubated in complete RPMI medium supplement with IL-2 (100 U/mL). Virus replication was measured by flow cytometry by quantifying intracellular HIV-1 Gag products (KC57-FITC antibody; Beckman Coulter) (Sáez-Ciri3n et al. 2010).

### **Metabolic drugs treatment**

Different compounds that target metabolic pathways: *2-deoxy-glucose*, *2-DG* (Sigma-Aldrich, Ref. D6134); *Orlistat* (Sigma, Ref. O4139); *6-diazo-5-oxo-l-norleucine*, *L-DON* (Sigma, Ref. D2141); *UK5099* (Sigma, Ref. PZ0160); *(+)-Etomoxir sodium salt hydrate* (Sigma, Ref. E1905); *BPTES* (Sigma, Ref. SML0601); *Metformin hydrochloride* (Sigma, Ref. M0605000); *Zaprinast* (Sigma, Ref. Z0878); *TOFA* (Sigma, Ref. T6575); *C75* (Sigma, Ref. C5490); *Haloperidol hydrochloride* (TOCRIS, Ref. 0931), were added to the culture media prior to infection at different concentrations depending on the protocol conditions.

### **Flow cytometry analysis**

After culturing, cells were stained with the LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific), for 15 minutes at room temperature, and then washed in PBS plus 1% FCS prior to staining with activation markers. For phenotype analyses, cells were additionally stained with anti-CD3-eFluor450 (clone SK7), anti-CD4-AlexaFluor700 (OKT4), anti-HLA-DR-FITC (G46-6), anti-CD25-ECD (B1.49.9), anti-CD69-PE-Cy7 (FN50), followed by incubation for 20 minutes at 4°C then washed twice with PBS plus 1% FCS and fixed in 1% paraformaldehyde for flow cytometry acquisitions on an LSRII-Fortessa device

(BD Bioscience). For infected samples, cells were stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, for 15 minutes at room temperature, and then washed in PBS plus 1% FCS prior to staining with surface markers anti-CD3-eFluor450 (clone SK7) and anti-CD4-AlexaFluor700 (OKT4). For surface GLUT1 labeling, CD4<sup>+</sup> T cells were stained with HRBD-rFc, a recombinant fusion protein that selectively binds GLUT1 (Metafora-biosystems, Paris, France), and a secondary goat-anti-Mouse Alexa Fluor 647 antibody (Thermofisher). For glucose uptake evaluation, cells were washed and prior to viability staining, incubated with 2-NBDG (2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose) (Thermo Fisher, Ref. N13195) at 75  $\mu$ M in PBS for 30 min at 37°C. Cells were treated with BD Transcription Factor Buffer Set (BD Biosciences) for intracellular anti-p24 staining. The data was analyzed with FlowJo software (TreeStar Inc).

### **Statistical analyses**

Statistical analyses were carried out with GraphPad Prism 9.5 software (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as medians. Single comparisons between two paired groups were performed with the Student's t-test. P-values <0.05 were considered to indicate statistically significant differences.



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## FIGURE LEGENDS

**Figure 1. The use of metabolic drugs downregulates CD4+ T cell activation markers; CD25, CD69 and HLA-DR.** The plotted values represent the frequency of CD4+ T cells that are positive for CD25, CD69 or HLA-DR flow cytometry staining. The values obtained for the treated conditions are normalized by the treatment free condition (untreated). **A.** CD4+ T cells treated with 5 mM 2-DG. **B.** CD4+ T cells treated with 4 uM Zaprinast. **C.** CD4+ T cells treated with 50 uM L-DON. **D.** CD4+ T cells treated with a 10 uM Orlistat. Each symbol represents results with cells from a different donor and the median values are indicated by horizontal lines.

**Figure 2. Several metabolic compounds impact the frequency of p24-positive CD4+ T cells 3 days after infection with HIV-1 BaL.** **A.** Frequency of CD4+ T infected cells in presence of 5 mM of 2-DG. CD4+ T cells from HIV-negative controls were activated, then infected with HIV-1 BaL in presence of 5 mM of 2-DG. (Left) Fold-change (normalized based on untreated condition) in p24-positive cell frequencies from 17 samples treated with 2-DG. (Right) An example of FlowJo gating to quantify the frequency of infected cells. Relative changes in the frequency of CD4+ T infected cells when cultured in presence of 5 uM 3-BrPA (n=6) (**B**), 4 uM Zaprinast (n=8) (**C**), 50 uM L-DON (n=11) (**D**), 10 uM BPTES (n=5) (**E**), 10 uM Orlistat (n=5) (**F**), 20 uM TOFA (n=5) (**G**), 50 uM C75 (n=8) (**H**), 10 uM ETO (n=5) (**I**) or 20 uM MET (n=6) (**J**). Infections were measured by flow cytometry (intracellular p24). Each symbol represents results with cells from a different donor and the median values are indicated by horizontal lines.

**Figure 3. Metabolic drugs cause the downregulation of the CD3<sup>hi</sup>CD4<sup>hi</sup> T cell population.** **A.** Representative flow cytometry plot of CD3 (Y-axis) and CD4 (X-axis) staining in live, untreated or cells treated with 5 mM 2-DG. **B.** Representative flow cytometry plot of CD3 (Y

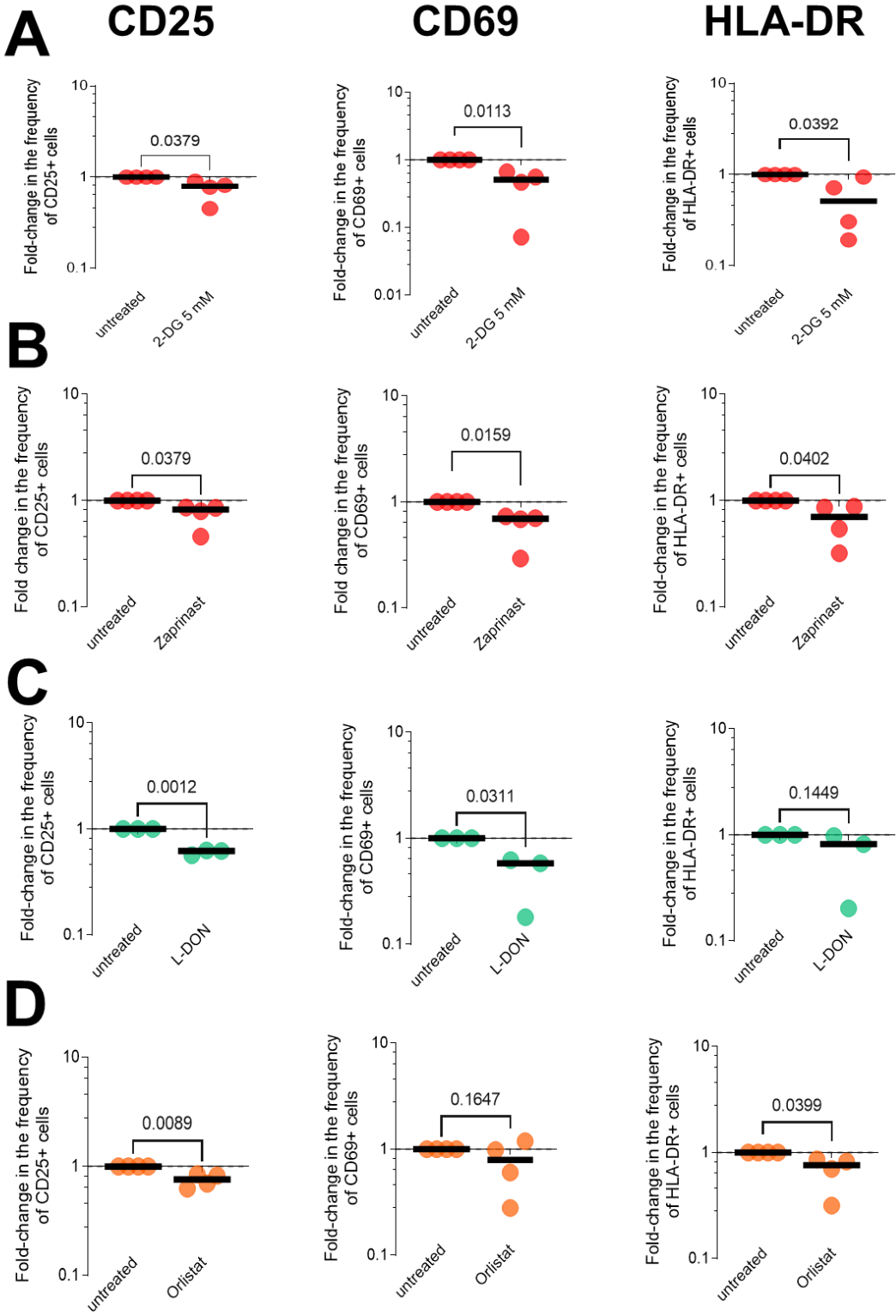
axis) and CD4 (X axis) staining in live, untreated CD4<sup>+</sup> T cells. Histograms representative of Glut1 MFI (left) and 2NBDG MFI (right), in CD3<sup>hi</sup>CD4<sup>hi</sup> (blue) and in CD3<sup>m</sup>CD4<sup>m</sup> (red) populations. **C.** The plotted values represent the fold-change of the frequency of CD3<sup>hi</sup>CD4<sup>hi</sup> in untreated uninfected CD4<sup>+</sup> T cells, and uninfected cells in treatment conditions as in Figure 2. Each symbol represents results with cells from a different donor and the median values are indicated by horizontal lines.

**Figure 4. Scatter plots showing the distribution of the infection levels according to the expression of activation markers.** Comparison of the changes in the frequency of CD25 (**A**), CD69 (**B**) and (**C**) HLA-DR-positive CD4<sup>+</sup> T cells vs changes in the frequency of p24-positive CD4<sup>+</sup> T cell provoked by treatment with metabolic drugs depicted in figures 1 and 2. **D.** Comparison of the changes in the frequency of CD3<sup>hi</sup>CD4<sup>hi</sup> population vs changes in the frequency of p24-positive CD4<sup>+</sup> T cell provoked by treatment with metabolic drugs depicted in figures 2 and 3.

**Figure 5. HIV-1 exposed CD4<sup>+</sup> T cells are more susceptible to metabolic drug-induced cytotoxicity.** **A.** Changes in the viability of non-infected (blue) and HIV-exposed (red) CD4<sup>+</sup> T cells treated with different concentrations of 2-DG (**A**), L-DON (**B**), Orlistat (**C**) 3-BrPA (**D**).

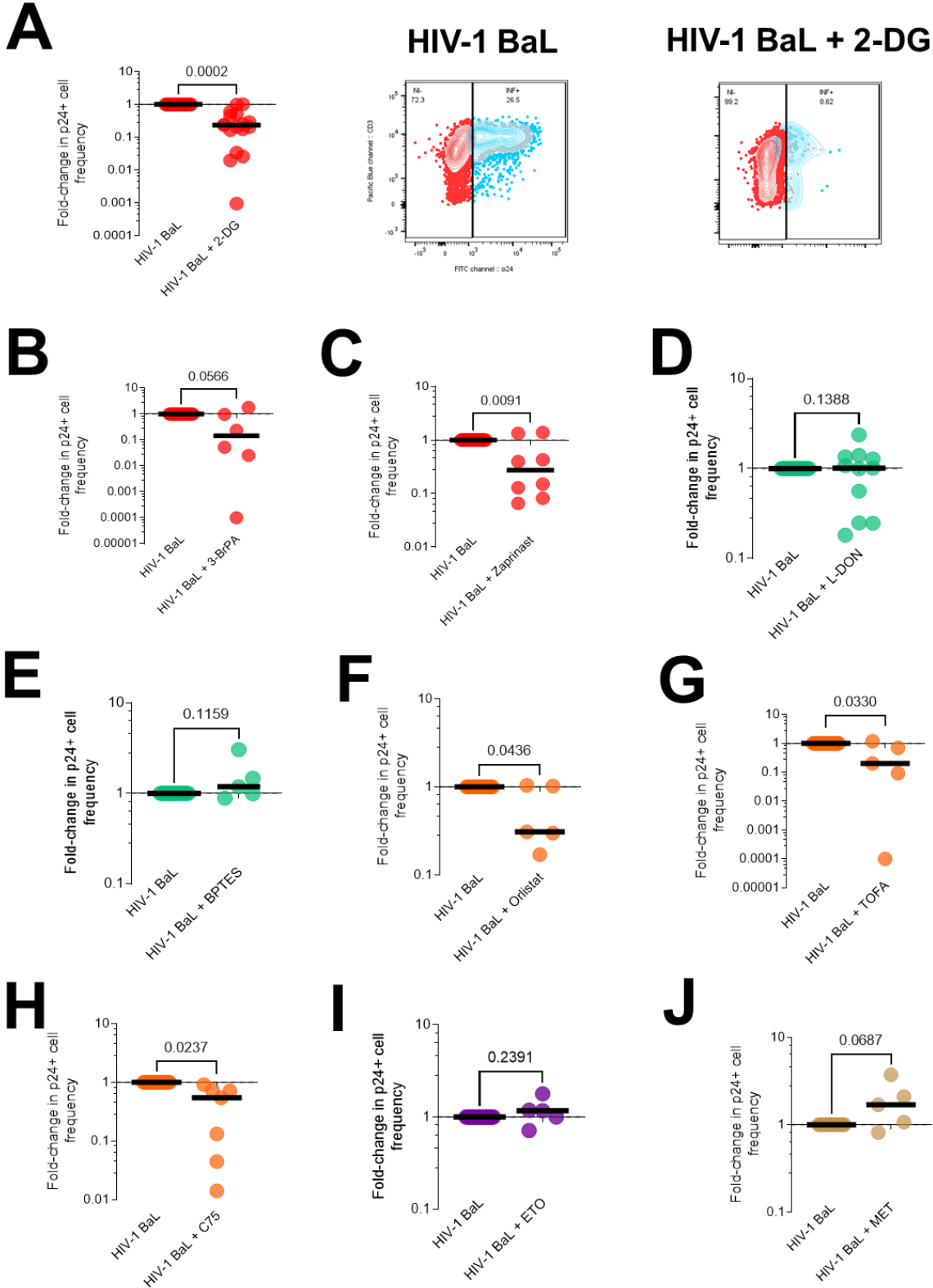


**Figure 1. The use of metabolic drugs downregulates CD4+ T cell activation markers; CD25, CD69 and HLA-DR.**



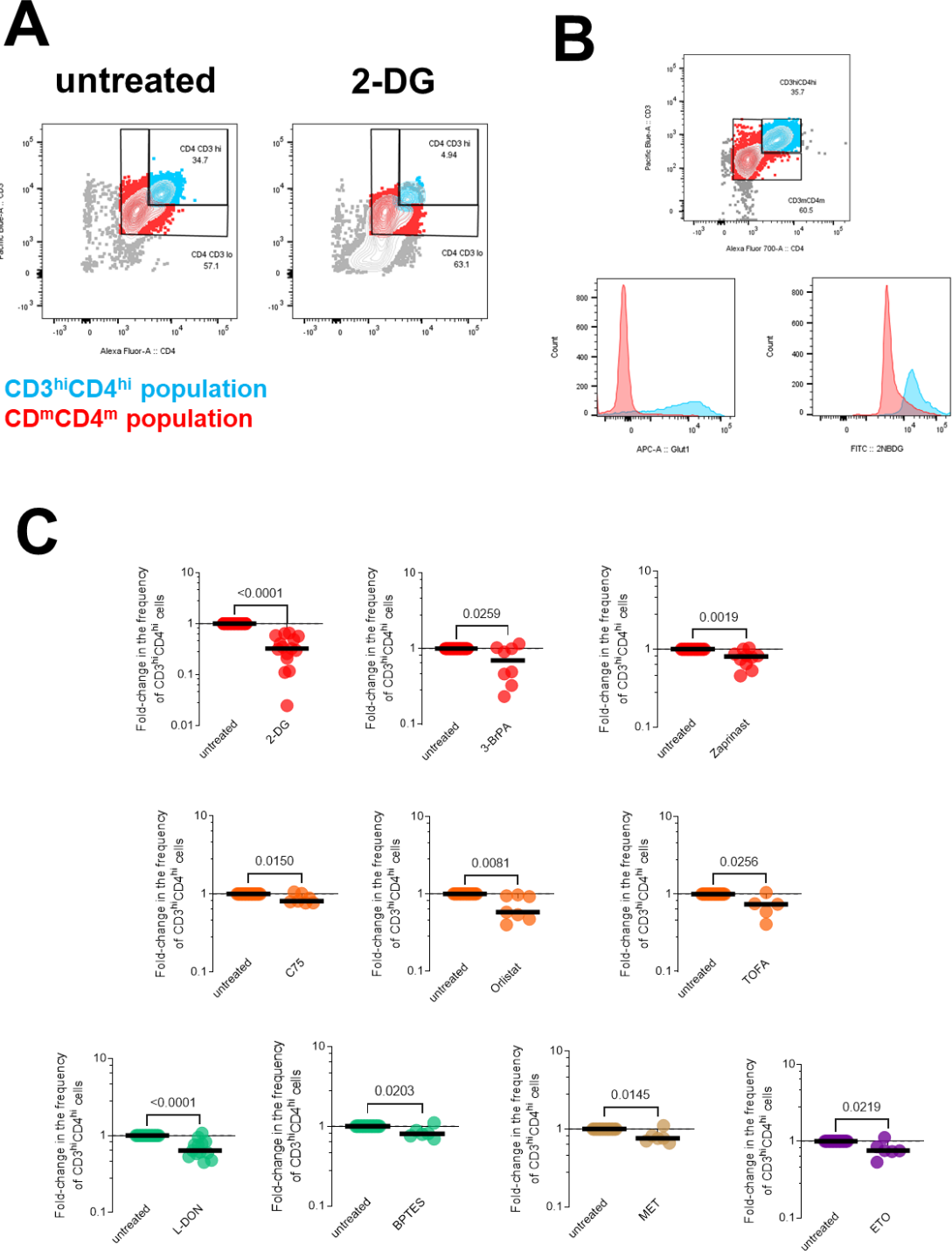
**Figure 1**

**Figure 2. Several metabolic compounds impact the frequency of p24-positive CD4+ T cells 3 days after infection with HIV-1 BaL.**



**Figure 2**

**Figure 3. Metabolic drugs cause the downregulation of the CD3<sup>hi</sup>CD4<sup>hi</sup> T cell population.**



**Figure 3**

Figure 4. Scatter plots showing the distribution of the infection levels according to the expression of activation markers.

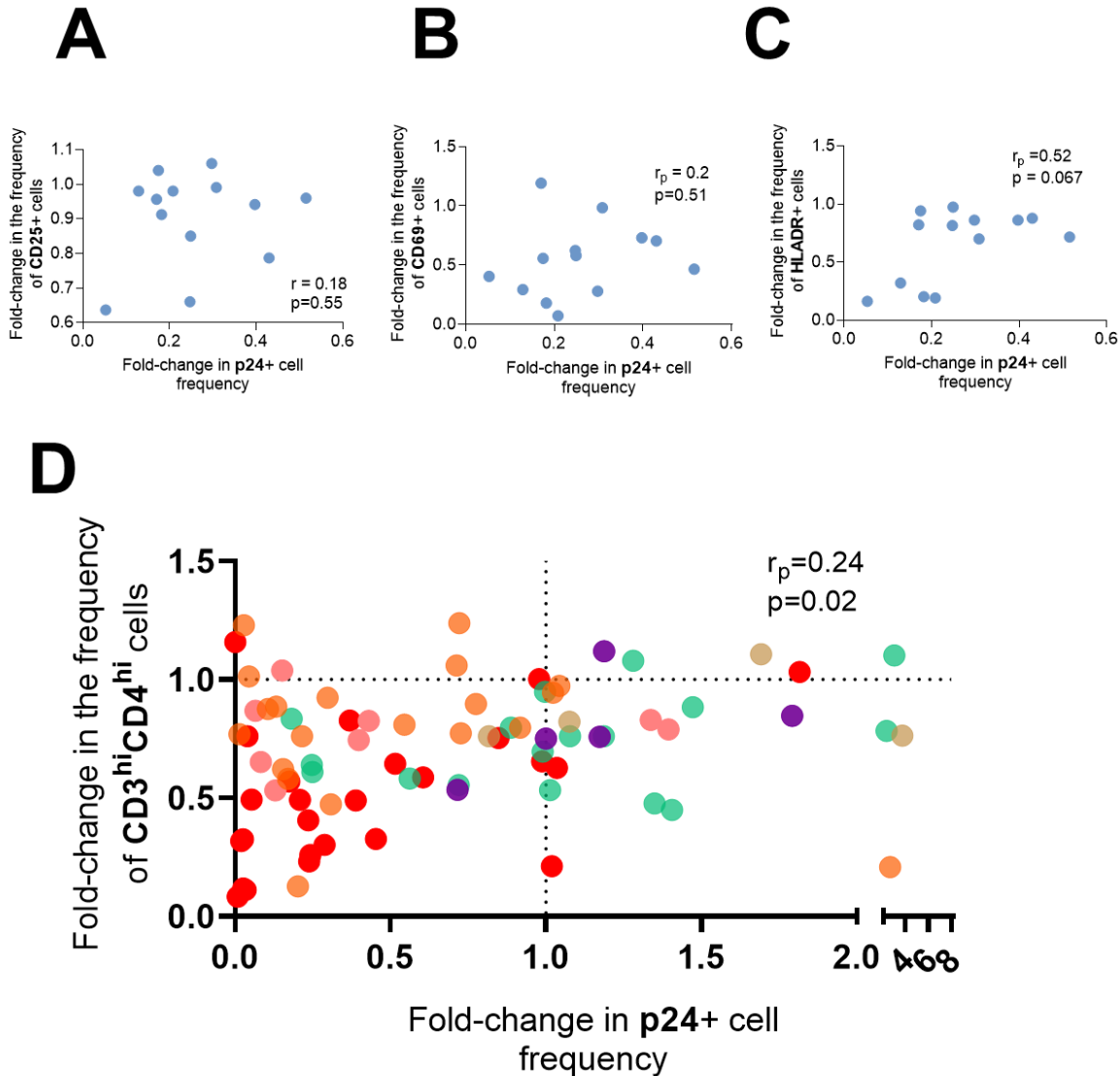
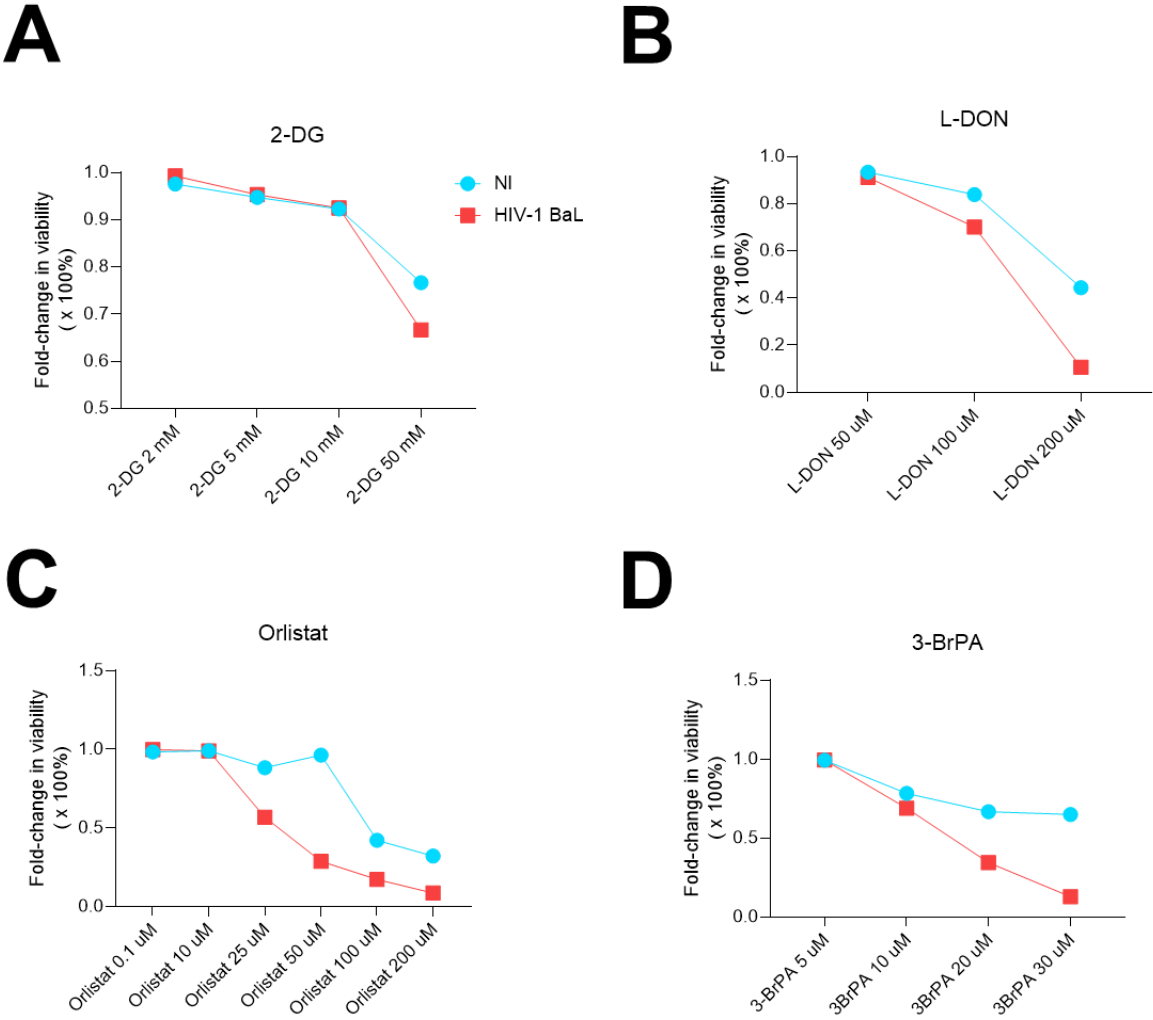


Figure 4

**Figure 5. HIV-1 exposed CD4+ T cells are more susceptible to metabolic drug-induced cytotoxicity.**



**Figure 5**

**CHAPTER II. DIFFERENTIAL EFFECT  
OF CYTOKINES ON CD4+ T CELL  
ACTIVATION, METABOLISM AND  
SUSCEPTIBILITY TO INFECTION**

## **Differential effect of cytokines on CD4+ T cell activation, metabolism and susceptibility to HIV-1 infection**

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### **Keywords**

HIV-1, CD4+ T cells, Immunometabolism, Metabolism, Cytokines, JAK/STAT,  $\gamma$ -chain cytokines, IL-10, IL-16, IFN- $\alpha$

## **ABSTRACT**

HIV-1 reservoirs are predominantly located in CD4<sup>+</sup> T cells, however not all CD4<sup>+</sup> T cells contribute to the reservoir in the same way. Factors such as activation, differentiation and cell metabolisms have been proposed to determine the relative susceptibility of cells to HIV-1 infection. HIV-1 reservoirs are seeded early during the acute phase of the infection, but the cell composition of these reservoirs evolves in the transition to chronic infection. This suggests that there are factors during the acute phase that may alter the intrinsic susceptibility of CD4<sup>+</sup> T cells to HIV-1. We investigated here the influence of common cytokines known to be secreted during the acute phase and to play a role either on T cell homeostasis or on HIV-1 infection on activation, differentiation, metabolic activity and HIV-1 susceptibility of CD4<sup>+</sup> T cells. We show that the pro-inflammatory cytokines IL-2, IL-7 and IL-15 induce cellular activation, differentiation to effector profile, increase in metabolic capacity and HIV-1 susceptibility. In contrast IL-21, another cytokine from the  $\gamma$ -chain family, showed opposing effects on the same parameters, including decreasing OXPHOS and HIV-1 infection. We also show that IL-10 and IFN- $\alpha$  are able to at least partially revert the cellular and metabolic changes induced by IL-2 or IL-7 and reduced the cells' susceptibility to HIV-1.



## INTRODUCTION

Despite the success of antiretroviral therapy (ART) in reducing HIV-1 replication and in preventing or reversing immunodeficiency in people living with HIV (PLWH), the persistence of HIV reservoir is a roadblock to an HIV cure. As such, integrated replication-competent proviruses in the reservoir cells drive a rapid viral rebound within weeks of ART cessation. This persistent reservoir is now the focus of extensive multinational research efforts. There are various types of HIV-harboring cells, however, it is well established that most of the HIV reservoir consists of CD4<sup>+</sup> T lymphocytes. Nevertheless, CD4<sup>+</sup> T lymphocytes are heterogeneous and the contribution of different CD4<sup>+</sup> T cell subsets to the reservoir varies (Brenchley et al. 2004; Chomont et al. 2009). The CD4<sup>+</sup> T cell compartment is regulated, not only by phenotype, but also by function, therefore forcing the distinction between a stable T cell lineage and a T cell differentiation state. In this regard, memory CD4<sup>+</sup> T subsets, such as stem-like memory cells (T<sub>SCM</sub>), central (T<sub>CM</sub>) and transitional (T<sub>TM</sub>) memory CD4<sup>+</sup> T cells, have been shown to be enriched in HIV proviral DNA. The heightened susceptibility to HIV infection, the survival and self-renewal capacities, and the inherent extended life span of many of these memory subsets provide the optimal environment for viral persistence.

Multiple studies have evaluated the potential clinical and immunological variables of HIV-1 reservoir size since PLWH with smaller HIV-1 reservoirs might be more amenable to cure. The formation of the HIV reservoir is estimated to take place during the acute HIV infection (AHI). Early ART initiation has been associated with a significant reduction in the size of the latent reservoir and a delay in viremia rebound upon treatment cessation (Buzon, Martin-Gayo, et al. 2014; Persaud et al. 2013; Strain et al. 2005). Further, the size of the reservoir appears to be only one piece of the puzzle, since early ART initiation was reported to reduce the contribution of T<sub>CM</sub> cells to the overall HIV reservoir, a trait observed in persons who naturally control HIV without ART. Post-treatment controllers (PTCs) are individuals that initiated ART during AHI

and demonstrate the ability to control viremia after ART cessation, show minimal  $T_{CM}$  contribution to the total HIV reservoir (Sáez-Cirión et al. 2013). This is consistent with other studies investigating the frequency of infection of distinct memory  $CD4^+$  T-cell subsets in participants who undertook ART early in infection (Buzon, Martin-Gayo, et al. 2014; Josefsson et al. 2013). Similarly, individuals able to control viremia in the absence of therapeutic interventions, referred to as long-term non-progressors (LTNPs), exhibit low  $T_{CM}$  cell infection levels (Descours et al. 2012).  $T_{SCM}$ , which share similar functional and self-renewal characteristics as  $T_{CM}$  cells, might also be an important HIV reservoir (Buzon, Sun, et al. 2014; Gattinoni et al. 2011). Interestingly, the contribution of this long-lived subset to the total HIV reservoir also may be considerably decreased by early ART initiation (Buzon, Martin-Gayo, et al. 2014). Given their crucial involvement in the first HIV immune responses, it is anticipated that the preservation of these long-lived memory subsets is of utmost relevance. In contrast, they are considered to be a great obstacle to the eradication of HIV due to their extended lifespan as compared to the short-lived effector subset.

During acute infection, when viral replication is most active, HIV reservoir is assumed to be largely composed of stored labile unintegrated DNA (Trémeaux et al. 2019). Long-lived populations (such as  $T_{CM}$ ) are believed to be more resistant to infection during this time, but as infection progresses to the chronic phase, they become more infection-permissive (Chéret et al. 2015). More crucially,  $T_{CM}$  cells contribute the most to the circulating HIV reservoir in people who are treated during the chronic phase of infection (Chomont et al. 2009; Soriano-Sarabia et al. 2014). Therefore, environmental factors such as cytokines released during AHI, which modulate the activation, differentiation and function of  $CD4^+$  T cells, may also influence their intrinsic permissibility to HIV-1 infection. During AHI, pro-inflammatory and adaptive cytokine levels are elevated and linked to plasma viremia (McMichael et al. 2009). Interestingly, profound dysregulation of inflammatory cytokine networks in blood, which

results in a proinflammatory environment, were demonstrated to enhance HIV-1 multiplication and transmission (McMichael et al. 2009; Olivier et al. 2014; Vanpouille et al. 2016).

We hypothesized that changes promoting CD4<sup>+</sup> T cell response and differentiation provide an adequate environment for HIV-1 replication since HIV-1 infection susceptibility varies with T cell differentiation, with effector memory cells being highly permissive and naive T cells being significantly resistant to infection (Buzon, Sun, et al. 2014; Chahroudi, Silvestri, and Lichterfeld 2015; Groot et al. 2006). Similarly, following activation by a cognate foreign antigen or cytokines, CD4<sup>+</sup> T lymphocytes become more permissive to HIV-1 infection and a multitude of studies showed that HIV infection of activated CD4<sup>+</sup> T cells is much more efficient than resting cells (Folks et al. 1986; Zack et al. 1988; Zagury et al. 1986). In fact, CD4<sup>+</sup> T cells exhibit fast clonal proliferation upon stimulation and it is well known that the intracellular environment linked to cell cycle entry favors HIV-1 infection. The chromatin environment of the integrated provirus and the transcriptional state of the CD4<sup>+</sup> T cell subset that carries latent HIV-1 have both been attributed to this unique intracellular milieu.

We have recently shown that HIV-1 preferentially infects CD4<sup>+</sup> T cells with high metabolic activity (Valle-Casuso et al. 2019). We proposed that the increased permissibility of CD4<sup>+</sup> T cells is controlled by modifications to the cellular metabolism, which drive the differentiation process. Indeed, metabolism is a key regulator of the biology of T cells. Engagement of the T cell receptor (TCR) and co-stimulatory signals sets off a metabolic switch that promotes protein, fatty acid, and nucleotide production, and biomass accumulation to promote cell proliferation and effector activities (Almeida et al. 2016). We showed that HIV-infected cells are characterized by high glycolysis and oxidative phosphorylation (OXPHOS). Furthermore, we demonstrated that suboptimal inhibition of glycolysis hampers the establishment of both productive and latent infection in CD4<sup>+</sup> T cells, as well as the reactivation and dissemination of the virus from the reservoir of PLWH.

Several studies have explored the involvement of cytokines, chemokines, and antigenic signals in shaping CD4<sup>+</sup> T cell activation and promoting HIV-1 infection. We explored here whether some of these cytokines may act by altering the default homeostatic CD4<sup>+</sup> T cell metabolic profile. We focused on cytokines known to be secreted early during acute infection, to have an impact of HIV-1 replication and or to play a significant role in CD4<sup>+</sup> T cell homeostasis. We studied specifically the effects of interleukin (IL)-2, IL-7, IL-10, IL-15, IL-16, IL-21 and type I interferon (IFN), in particular IFN- $\alpha$ 2a (referred to as IFN- $\alpha$  in this manuscript). The common  $\gamma$ -chain ( $\gamma$ c) cytokines IL-2, IL-7, IL-15 and IL-21 are involved in T cell homeostasis, development, expansion and differentiation. IL-21 is associated with T<sub>FH</sub> and T<sub>H17</sub> differentiation, two of the preferential targets of HIV infection (R. Nurieva et al. 2007; R. I. Nurieva et al. 2008). IL-10 is a regulatory cytokine that has been shown to drive T<sub>REG</sub> cell response in HIV infection and is associated with viral persistence on ART (Harper et al. 2022). IL-7 and the pro-inflammatory cytokines IL-2 and IL-15 have been rather associated with an increased HIV-1 replication, whereas the anti-inflammatory IL-10 and IFN- $\alpha$ 2a were shown to inhibit HIV-1 replication. IL-16 shares the main HIV-1 receptor, CD4 and has been described to inhibit its replication. We investigated *in vitro* the impact of these immunomodulators on CD4<sup>+</sup> T cell activation, differentiation, metabolism and susceptibility to HIV-1 infection.

## RESULTS

### Divergent effect of cytokines on CD4<sup>+</sup> T cell activation and differentiation

We first assessed the impact of IL-2, IL-7, IL-10, IL-15, IL-16, IL-21 and IFN- $\alpha$  on the expression of several markers of activation and differentiation on purified CD4<sup>+</sup> T cells from blood donors. As expected, IL-2, IL-7 and IL-15 strongly activated CD4<sup>+</sup> T cells, as shown by the upregulation of CD25, CD69, HLA-DR, PD-1, CD95 and Ki67 after 5 days of culture in presence of these cytokines when compared to the non-stimulated condition (Figure 1A, Figure S1). In contrast, only a small increase in CD25 and PD-1 was induced by IL-21, and no significant effect on CD4<sup>+</sup> T cell activation was observed for IL-10, IL-16 or IFN- $\alpha$  when compared to the control condition (Figure 1A, Figure S1). The changes in the activation markers induced by the different cytokines were accompanied by equivalent changes in CD4 expression levels and in the size and complexity of the cells (Figure S1).

Accordingly, IL-2, IL-7 and IL-15 promoted T cell differentiation (Figure 1B), which was evidenced by a decrease in the proportion of CD4<sup>+</sup> T cells with a CM and EM profile and an increase of cells with an effector profile (T<sub>EMRA</sub>) (Figure S2). In contrast, IL-10 and IL-21 promoted a memory phenotype, increasing the levels of CCR7 (Figure 1B) and decreasing the proportion of cells with T<sub>M</sub> and T<sub>EMRA</sub> profile, when compared with the non-stimulated condition. A decrease in the proportion of T<sub>EMRA</sub> CD4<sup>+</sup> T cells was also observed in presence of IFN- $\alpha$ . Overall, in our experimental conditions, IL-7 was the cytokine that most efficiently stimulated CD4<sup>+</sup> T cells, followed by IL-2 and IL-15, which had equivalent effects (Figure 1C, D). This was likely related to the more frequent constitutive presence of IL7R/CD127 among CD4<sup>+</sup> T cells (Figure S3). IL-10, IL-21 and IFN- $\alpha$  did not have much effect on T cell activation but restrained T cell differentiation, while IL-16 had a more neutral effect.

### **Different impact of $\gamma$ c-cytokines on glycolysis and oxidative phosphorylation**

We next determined the impact of our panel of cytokines on the metabolic activity of CD4<sup>+</sup> T cells. We used a cell flux analyzer to measure the oxygen consumption rate (OCR) and the proton efflux rate (PER) as indicators of OXPHOS and glycolysis in the cells, respectively. IL-7, IL-2 and IL-15 strongly increased the basal glycolytic activity of the cells and their maximal glycolytic capacity (Figure 2A, Figure S4). IL-16 and IL-21 slightly increased the glycolytic activities of the CD4<sup>+</sup> T cells. IL-7 strongly enhanced also the basal respiratory activity and the maximal respiratory capacity of CD4<sup>+</sup> T cells (Figure 2B). Enhancing effects were observed for IL-2 and IL-15 in most but not all the samples analyzed (Figure 2B). We observed that while IL-7 equally enhanced the maximal respiratory and glycolytic capacity of the cells, IL-2 appeared to preferentially enhance the maximal glycolytic capacity (Figure 2C), which reached similar levels on cells cultured in presence of IL-2 and IL-7 (Figure 2A). In contrast, IL-21 and IFN- $\alpha$  decreased the basal respiratory capacity of the CD4<sup>+</sup> T cells (Figure 2B). A reduction in the maximal respiratory capacity was also observed in cells cultured in presence of IL-21 when directly compared to the control condition ( $p=0.04$ ), but statistical significance was lost in the multiple comparison analysis (Figure 2B). Overall, these results suggest contrasting effects on CD4<sup>+</sup> T cell metabolism of IL-21 and the other  $\gamma$ c-cytokines analyzed.

To further explore these differences, we analyzed the expression of a panel of genes related to the regulation of different metabolic pathways (Table S1) in CD4<sup>+</sup> T cells from three donors cultured in absence or presence of different cytokines (Figure 2D). We found that while IL-7 strongly increased the expression of *ENO1* (Enolase 1) and *IDH2* (isocitrate dehydrogenase (NADP(+)) 2), two key enzymes involved in glycolysis and the tricarboxylic acid cycle, respectively (Figure 2D), IL-21 increased the expression of *FOXO1* (Forkhead Box O1) and *PRKAB1* (5'-AMP-activated protein kinase subunit beta-1), which are regulators of cell

metabolism, and *SLC3A2* (Solute Carrier Family 3 Member 2) and *KYAT1* (Kynurenine Aminotransferase 1) which are involved in the regulation of the kynurenine pathway. These results suggest that IL-7 and IL-21 mobilized different metabolic pathways to sustain the activities of CD4<sup>+</sup> T cells.

### **Contrasting effect of cytokines on the susceptibility of CD4<sup>+</sup> T cells to HIV-1 infection**

HIV-1 infection has been proposed to increase with the activation status and the metabolic activities of CD4<sup>+</sup> T cells. We therefore analyzed the effect of the cytokines studied here on the susceptibility of CD4<sup>+</sup> T cells to infection with replication competent HIV-1 BaL. We found that IL-2, IL-15 and particularly IL-7 enhanced the frequency of HIV-1 infected cells when compared to the non-stimulated condition (Figure 3). In contrast, the number of infected cells was reduced when the cells were cultured in the presence of IL-21 or IFN- $\alpha$ , resembling the effects that were observed for the basal mitochondrial activity. No significant effects were observed for IL-10 or IL-16 in these conditions.

### **IL-10 antagonized the enhancing effects of IL-2 and IL-7 on CD4<sup>+</sup> T cell metabolic activity and susceptibility to HIV-1 infection**

We wondered whether the potentially repressing effect of some cytokines such as IFN- $\alpha$  or IL-10 might have been masked by the relative quiescence of the CD4<sup>+</sup> T cells that we used *ex vivo*. We therefore explored whether these cytokines could counteract the enhancing effects that were observed when cells were cultured in presence of IL-2 and/or IL-7. We found that whereas the absence or presence of IFN- $\alpha$  did not alter the metabolic activities of CD4<sup>+</sup> T cells cultured with IL-2 (Figure 4A), the additional presence of IFN- $\alpha$  dampened the metabolic activities, notably the respiratory capacity, of the cells that were cultured in the presence of IL-7 (Figure 4B).

Conversely, cells cultured in the presence of both IL-2 and IL-10 showed lower glycolytic activity and potential than cells cultured in the presence of IL-2 alone, and a trend was also observed towards a decrease in OXPHOS in the presence of IL2+IL-10 vs IL2 alone (Figure 4C). The presence of IL-10 did not alter the glycolytic capacity of cells that were cultured in the presence of IL-7 but did decrease their basal respiratory activity and maximal respiratory capacity (Figure 4D). Moreover, the presence of IL-10 decreased the frequency of HIV-1 infected cells that were observed when CD4+ T cells were cultured with IL-2 or IL-7 alone (Figure 4E).

These results indicate that cytokines may show different antagonizing effects on CD4+ T cell metabolic activities. While IL-2 appears more sensitive to the antagonizing effects of IL-10 than to IFN- $\alpha$ , the mitochondrial activities enhanced by IL-7 are reduced in the presence of both IL-10 and IFN- $\alpha$ .



## DISCUSSION

In this study, we have compared the effects of several cytokines on CD4<sup>+</sup> T cell activation, differentiation, metabolism, and susceptibility to HIV-1 infection *in vitro*. The common  $\gamma$ -chain cytokines IL-2, IL-7, and IL-15, which play essential roles in peripheral T cell growth and survival, exhibited, as expected, comparable enhancing effects on all markers of cellular activation. This increase in cell activation was accompanied by a significant decline in both the T<sub>CM</sub> and T<sub>EM</sub> subsets while increasing the T<sub>EMRA</sub> subset. This is in agreement with previous studies showing that in the absence of antigenic stimulation T<sub>CM</sub> cells evolve into CD45RA<sup>+</sup>CCR7<sup>-</sup> T<sub>EMRA</sub> cells in the presence of IL-2, IL-7, and IL-15, emphasizing the importance of these cytokines as factors in the development and maintenance of the functional effector CD4<sup>+</sup> T cell response (Geginat, Lanzavecchia, and Sallusto 2003; Kondrack et al. 2003). IL-2, IL-7 and IL-15 share one receptor subunit and a common signaling pathway through STAT5 (Rochman, Spolski, and Leonard 2009). As a result, these cytokines are likely to have comparable effects. In our study, IL-7 showed the strongest effect when compared to IL-2 and IL-15, which might be explained in part by the higher constitutive expression of IL-7Ra (CD127) by CD4<sup>+</sup> T in the absence of stimulation, when compared to IL-2Rb (CD122), a receptor component shared by IL-2 and IL-15 (Kim, Imbert, and Leonard 2006; Schluns et al. 2000; Waldmann 2006). However, it is important to take into account that each of these cytokines has subtle differences in their stimulating capacities that promote particular functions. In our study, this was better observed at the level of cell metabolism.

IL-2, IL-7, and IL-15 increased the cells' overall metabolic activity. However, whereas IL-7 demonstrated a distinct profile significantly increasing glycolysis and OXPHOS, IL-2 and IL-15 enhanced the basal and maximum glycolysis in cells but the effects were less marked for mitochondrial respiration. Moreover, IL-2 increased the maximal glycolytic capacity of CD4<sup>+</sup> T cells to levels comparable to those observed for IL-7. Taken together, our findings suggest

that IL-2 provoked a more glycolysis-skewed increase of cell metabolism while IL-7 induced a more balanced increase of glycolysis and OXPHOS capacities. IL-2, IL-7 and IL-15 may increase glycolysis through antagonizing Bcl-6, a glycolysis suppressor, whose expression is inhibited by STAT5 signaling (Liu et al. 2016; McDonald et al. 2016). In addition, IL-2, IL-15, and, to a lesser extent, IL-7 stimulate mTORC1 activation in CD4<sup>+</sup> T cells (Marzec et al. 2008). mTORC1 is known to stimulate glycolysis, which may explain why the relative increase in glycolysis in CD4<sup>+</sup> T cells treated with IL-2 was higher than in IL-7. On the other hand, IL-7 has also been shown to improve glycerol uptake for triglyceride synthesis and storage which provides substrates for FAO (Cui et al. 2015). This implies that IL-7 mobilizes FAO as well as glycolysis to fuel the TCA cycle, explaining the high increase in OXPHOS (Vignali et al. 2018). Notably, IL-15 also promotes FAO by increasing the expression of CPT1A, which catalyzes the transfer of the acyl group of long-chain fatty acid-CoA conjugates onto carnitine, an essential step for the mitochondrial uptake of long-chain fatty acids and their subsequent  $\beta$ -oxidation in the mitochondrion. This engages the TCA cycle, and IL-15 has been demonstrated to condition the cell's OXPHOS capacity by stimulating mitochondrial fusion and enhancing the mitochondrial spare respiratory capacity of CD8<sup>+</sup> T cell. The lesser effect of IL-15, when compared to IL-2 and/or IL-7, in our experiments could be related to the low expression on CD4<sup>+</sup> T cells of IL-15R $\alpha$  (CD215), which together with  $\gamma c$  and IL-2R $\beta$  constitute the high affinity receptor for this cytokine. In addition, cytokines use non-canonical pathways that differ from one another in addition to canonical pathways (JAK/STAT pathway) (Johnson et al. 2013). This ensures cell specificity and metabolic plasticity in some cells.

Despite the subtle differences in the cell profiles promoted by the three cytokines, all enhanced the susceptibility of CD4<sup>+</sup> T cells to HIV-1 infection. Several studies have found that IL-2, IL-7, and IL-15 increase resting CD4<sup>+</sup> T cell susceptibility to HIV-1 (Unutmaz et al. 1999). Glycolysis-mediated expansion of the dNTP pool accessible for viral reverse transcriptase

activity, via synchronized increases in nutrient uptake and nucleotide biosynthesis pathways, and second, increased acetyl-CoA abundance, leading to acetylation/stabilization of microtubules and allowing cytoplasmic transport of viral products, were proposed as the molecular foundation for this. (Coiras et al. 2016; Manganaro et al. 2018; Taylor et al. 2020). Importantly, inhibiting not only PI3K signaling but also GLUT1 signaling abolished this impact (Loisel-Meyer et al. 2012; Palmer et al. 2017).

IL-21, another cytokine from the  $\gamma c$  family, had very contrasting effects when compared to IL-2, IL-7 and IL-15. A common feature of  $\gamma c$  cytokines is the upregulation PD-1 on purified T cells in vitro (Kinter et al. 2008), and we did observe an upregulation of PD-1 expression and CD25 upon treatment with IL-21, but no significant effect on CD69, HLA-DR, CD95, or Ki67 levels. Although IL-21 shares the  $\gamma c$  receptor subunit with the other members of the family, IL-21 primarily engage STAT3, and not STAT5, which might explain why it had a more comparable effect to IL-10, which also engages STAT3, than to the other cytokines in the  $\gamma c$  family. IL-10 did not show any discernible effect on CD4<sup>+</sup> T cell activation in our conditions, but both IL-10 and IL-21 preserved the frequency of the memory subsets, maintaining CCR7 expression, and decreasing the contribution of the more differentiated T<sub>TM</sub>, T<sub>EM</sub> and T<sub>EMRA</sub> to the pool of CD4<sup>+</sup> T cells. IL-21 has been shown to enhance T<sub>SCM</sub> frequencies in T cells when combined with a short anti-CD3/CD28 co-stimulation (Alvarez-Fernández et al. 2016) which is agreement with our data. Although IL-21 caused a very modest increase in basal and maximal glycolysis capacity, it led to a significant reduction in OXPHOS. This shows that IL-21 regulates these major metabolic pathways differently from IL-2, IL-7, and IL-15. Contrasting effects on the metabolic activity of IL-2 and IL-21 have already been reported for CD8<sup>+</sup> T cells (Hermans et al. 2020). Our results suggest an upregulation of the kynurenine pathway may be favored by IL-21. We could not observe any impact of IL-10 on the metabolic activity of CD4<sup>+</sup> T cells when added to non-stimulated cells. In contrast, IL-10 inhibited the metabolic

enhancement caused by IL-2 and IL-7. More specifically, IL-10 appeared to more decisively reduce the IL-2-induced increase in glycolysis, while it only dampened the IL-7-induced increase in mitochondrial activity. Therefore, IL-2 and IL-7 stimulation was differently counteracted by IL-10, further showing that these cytokines did not activate the same metabolic response.

We found that IFN- $\alpha$ , a cytokine that participates in the regulation of T cell response through direct signaling (Crouse, Kalinke, and Oxenius 2015; Nguyen et al. 2015), did not provoke major changes in T cell activation and differentiation, except decreasing the proportion of effector cells and CD4 and CD27 levels in most of the samples analyzed. IFN- $\alpha$  did not alter the glycolytic activities of CD4<sup>+</sup> T cells but decrease their basal respiratory activity. Moreover, IFN- $\alpha$  did not counteract the metabolic changes induced by IL-2 (biased towards glycolysis), but decreased the metabolic activities induced by IL-7, in particular the basal and maximal respiratory capacities. These results suggest that IFN- $\alpha$  is involved in the regulation of oxidative phosphorylation in CD4<sup>+</sup> T cells. The impact of type I interferon on T cell metabolism is increasingly recognized (Stephanie Deborah Fritsch 2016), but remains unclear. Decrease mitochondrial activities have been observed in CD4<sup>+</sup> T cells from persons with multiple sclerosis treated with IFN- $\beta$  (Haghikia et al. 2015) and IFN- $\alpha$  was reported to block IL-7 induced proliferation of CD4<sup>+</sup> T cells by inhibiting the induction of p-AKT (Nguyen et al. 2015).

The capacity of IL-10, IL-21 and IFN- $\alpha$  to downmodulate the metabolic activities of CD4<sup>+</sup> T cells was accompanied by a capacity of these cytokines to reduce the susceptibility of the cells to HIV-1 infection, either directly or dampening the enhancing effects of IL-2/IL-7. Although we have not demonstrated a direct association between these effects, we and others have previously shown that the specific inhibition of metabolic activities in CD4<sup>+</sup> T cells blocks HIV-1 infection (Clerc et al. 2019; Valle-Casuso et al. 2019). It is therefore tempting to propose

that the capacity of the cytokines evaluated here to enhance or repress cell metabolism may contribute to shape their susceptibility to HIV-1 infection. Moreover, our results suggest that the capacity of IFN- $\alpha$  to rewire the CD4<sup>+</sup> T cell metabolic program could reinforce the refractory activities of interferon-induced antiviral factors.

Our study provides a comparative overview of the complex effects that cytokines have on T cell activation, differentiation and cell metabolism and their possible impact on their susceptibility to HIV-1 infection. Although it is generally considered that metabolism, activation and differentiation are intertwined processes, our results show that, although generally related, they are not immediately equivalent and are subtly regulated, leading to differences that may affect the sensitivity of the cells to complementary or antagonistic signaling. Our results indicate that the “cytokine storm” that occurs during acute HIV-1 infection may decisively influence the fate of CD4<sup>+</sup> T cells in terms of HIV-1 infection and the establishment of viral reservoirs.

## **METHODS**

### **Isolation and culture of primary human CD4<sup>+</sup> T cells**

Primary human cells were used in all of the experiments. As part of an agreement with the Institut Pasteur (C CPSL UNT, number 15/EFS/023), blood samples from volunteer donors were collected from the French blood bank (Établissement Français du Sang).

Peripheral blood mononuclear cells (PBMCs) were isolated from donor-derived buffy coats. Buffy coats were diluted 1:1 (vol/vol) in a phosphate-buffered saline (PBS) buffer, overlaid on a density gradient medium, Lymphocyte Separation Medium (EuroBio) at a ratio of 2:1 blood to Ficoll and then centrifuged at 1,800 rpm for 30 min at minimum acceleration/deceleration to harvest PBMCs. Red blood cells (RBC) were removed using 1X RBC lysis buffer.

CD4<sup>+</sup> T cells were then purified from the PBMCs by negative selection using Stemcell EasySep Human CD4<sup>+</sup> T cell enrichment kit. CD4<sup>+</sup> T cells-enriched PBMCs show purities greater than 90%. Cells were cultured at a density of 10<sup>6</sup> cells/ml in RPMI 1640 containing Glutamax (ThermoFisher), supplemented with 10% fetal calf serum (FCS), penicillin (10 IU/mL) and streptomycin (10 µg/mL) (referred to below as culture medium). Cells are then maintained at 37°C in a standard tissue culture humidified incubator containing atmospheric (20%) O<sub>2</sub> and 5% CO<sub>2</sub>.

### **Infection of primary CD4<sup>+</sup> T cells**

Activated CD4<sup>+</sup> T cells were infected with HIV-1 BaL (R5) (7 ng/ml p24) virus by centrifuging at 1,200 × g for 1 h at room temperature and then incubating for 1 h at 37°C in a humidified 5% CO<sub>2</sub> incubator (O'Doherty, Swiggard, and Malim 2000). The cells were then washed once with PBS and incubated in culture medium. Infection levels were evaluated on day 3 post-infection using flow cytometry, by HIV-1-Gag protein, p24 intracellular staining (p24-FITC (clone KC57, Coulter) (Sáez-Cirión et al. 2010).

### **Activation phenotyping**

Cells were first stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, for 15 minutes at room temperature, then washed then washed in PBS plus 1% FCS prior to staining with activation markers. Cell activation was monitored using fluorochrome-conjugated monoclonal antibodies (mAbs) (from BD Bioscience, Beckman Coulter, Biolegend, Thermofischer or Milteyni). We used the following antibodies:

- **CD3**-eFluor450 (clone SK7),
- **CD4**-AlexaFluor700 (OKT4),
- **HLA-DR**-FITC (G46-6),
- **CD25**-ECD (B1.49.9),
- **CD69**-PE-Cy7 (FN50),
- **PD-1**-PE (eBioJ105),

CD4<sup>+</sup> T cells were incubated with the surface markers antibodies for 20 minutes at 4°C then washed twice in PBS plus 1% FCS. For the intracellular staining, cells were treated with BD Cytotfix/Cytoperm (from BD Biosciences) solution for 20 minutes at 4°C. After two washing steps with 1X BD Perm/Wash solution, **Ki67**-eFluor660 (20Raj1) was added to the cells for 30 minutes at 4°C. Finally, cells were washed twice in PBS plus 1% FCS and fixed in 1% paraformaldehyde for flow cytometry acquisitions on an LSRII-Fortessa device (BD Bioscience). The data was analyzed with FlowJo software (TreeStar Inc).

### **Differentiation phenotyping**

Cells were first stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, for 15 minutes at room temperature, then washed then washed in PBS plus 1% FCS prior to staining with activation markers. Immunophenotyping was monitored using fluorochrome-conjugated mAbs (from BD Bioscience, Beckman Coulter, Biolegend, Thermofischer or Milteyni). We used the following antibodies:

- **CD3**-eFluor450 (clone SK7),
- **CD4**-AlexaFluor700 (OKT4),
- **CD45RA**-PE-Cy7 (5H9),
- **CCR7**-PE-Dazzle594 (G043H7),
- **CD95**-PerCP Cy5.5 (DX2),
- **CD27**-APC (MT271)

CD4<sup>+</sup> T cells were incubated with the antibodies for 20 minutes at 4°C then washed twice in PBS plus 1% FCS and fixed in 1% paraformaldehyde for flow cytometry acquisitions on an LSRII device (BD Bioscience). The data was analyzed with FlowJo software (TreeStar Inc).

The different CD4<sup>+</sup> T-cell subsets were defined as follows:

- Naïve or **T<sub>N</sub>** ; CD3<sup>+</sup>, CD4<sup>+</sup>, CD45RA<sup>+</sup>, CCR7<sup>+</sup>, CD27<sup>+</sup>, CD95<sup>-</sup>,
- Stem cell memory or **T<sub>SCM</sub>** ; CD3<sup>+</sup>, CD4<sup>+</sup>, CD45RA<sup>+</sup>, CCR7<sup>+</sup>, CD27<sup>+</sup>, CD95<sup>+</sup>,
- Central memory or **T<sub>CM</sub>** ; CD3<sup>+</sup>, CD4<sup>+</sup>, CD45RA<sup>-</sup>, CCR7<sup>+</sup>, CD27<sup>+</sup>,

- Transitional memory or **T<sub>TM</sub>** ; CD3+, CD4+, CD45RA-, CCR7-, CD27+,
- Effector memory or **T<sub>EM</sub>** ; CD3+, CD4+, CD45RA-, CCR7-, CD27-

### **Measurements of cellular metabolism**

Following incubation, live cells were counted using an automated Countess cell counter (Invitrogen) based on size and trypan blue non-staining. The number of living cells was then normalized before analysis. Briefly, cells ( $2.5 \times 10^5$ ) were resuspended in Seahorse XF RPMI medium, supplemented with sodium pyruvate (1 mM) and L-glutamine (2 mM) in the presence or absence of glucose (10 mM), and added to Cell Tak-coated wells (22,4 µg/ml, Corning). Prior to loading the plate into the Seahorse analyzer, the cells were incubated for 50 minutes in a CO<sub>2</sub>-free incubator at 37°C. Depending on the assay, different programs were performed on the Seahorse analyzer. Mitochondrial function was analysed by performing OCR and PER measurements, before a subsequent three measurements following each compound injection. Drug Panel A; (1) XF RPMI medium 2) oligomycin (2.5 µM), 3) FCCP (0.9 µM) and 4) rotenone and antimycin A (1 µM)) were delivered through ports A, B, C and D, respectively, for the mitochondrial stress test. Drug panel B (1) XF RPMI media 2) rotenone and antimycin A (0.5 µM), and 3) 2-DG (50 mM)) was used for the glycolytic rate assay.

Basal OCR and PER are the mean values of the three baseline measures from three replicated (a mean of nine values in total). Maximal OCR and PER capacities were calculated as the mean value of three corresponding measurements from three replicates following injections of FCCP, oligomycin, or rotenone/antimycin, respectively.

### **Quantitative RT-PCR Arrays**

After culturing, cells were counted, and a total of 20,000 cells per condition were placed in 96-well plates containing VILO Reaction Mix, SUPERase-In, and NP40 (all from ThermoFisher Scientific). RNA was denatured at 65°C for 90 sec and then snap-chilled on ice. Reverse transcription was performed with the 10X Superscript Enzyme Mix (Thermo Fisher Scientific), and T4 Gene 32 Protein (Merck), using the following cycling: 25°C: 5 min; 50°C: 30 min; 55°C: 25 min; 60°C: 5 min; 70°C: 10 min. Specific target amplification (STA) was performed by adding TaqMan PreAmp Master Mix (ThermoFisher Scientific), 10X 96 primer mix, and 0.5 EDTA (Thermo Fisher Scientific) to complementary cDNA, using the following cycling: 96°C: 10 min; 20 cycles of 96°C: 5 sec and 60°C: 4 min. The samples were then treated with exonuclease I (New England BioLabs) at 37°C: 30 min and 80°C: 15 min. All samples were 5-fold diluted in 1X DNA suspension buffer (TEKnova). Exonuclease I-treated, diluted samples were mixed with 2X SsoFast EvaGreen Supermix with low ROX (Bio Rad), and 20X DNA



Binding Dye (Fluidigm), whereas the sets of Delta Gene primers (Fluidigm, Table S1) were mixed with 2X Assay Loading Reagent (Fluidigm), and 1X DNA Suspension Buffer, and then loaded on a primed 96.96 Dynamic Array chip (Fluidigm cat: BMK-M-96.96). The chip was thermocycled in a Biomark (Fluidigm) and fluorescence was measured using the GE 96 x 96 PCR+ Melt v.2 software. The baseline correction for linear derivative mode was used. We used the web-based comprehensive tool RefFinder to rank the stability of our housekeeping gene candidates (Xie et al. 2012). RefFinder integrates four computational programs; geNorm (Vandesompele et al. 2002), Normfinder (Andersen, Jensen, and Ørntoft 2004), BestKeeper (Pfaffl et al. 2004), and the comparative  $\Delta C_t$  method (Silver et al. 2006) to compare and rank the tested candidate reference genes. Based on the expression stability in the investigated samples, IPO8 was determined as the best normalizing gene (Figure S5), and the gene expression levels were plotted using  $2^{-\Delta\Delta C_t}$ , where  $\Delta\Delta C_t = \Delta C_{t\text{SAMPLE}} - \Delta C_{t\text{CONTROL}}$ , and  $\Delta C_t = C_{t\text{TARGET GENE}} - C_{t\text{IPO8}}$ .

### **Statistical analyses**

Data were analyzed using GraphPad Prism software, version 9 (GraphPad Prism, La Jolla, CA). Data are presented as medians and ranges. Non parametric one-way analysis of variance (ANOVA) was used to assess the changes observed in the different cytokine conditions relative to the non-stimulated controls. Post-hoc analyses were done using the Benjamini, Krieger, Yekutieli method to correct for multiple comparisons (corrected p values are shown). The ratio paired T test was used for the comparison of paired relative data in two experimental conditions. p values of less than 0.05 were considered statistically significant. Heat map and PCA analyses were done using Qlucore Omics Explorer v3.8 setting a p value < 0.5 for multigroup comparisons and eliminating the sample ID as a confounding factor.

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## FIGURE LEGENDS

**Figure 1. IL-2, IL-7 and IL-15 increase cellular activation and differentiation of CD4+ T cells.** Relative changes in the expression levels of CD25, CD69, HLADR, Ki67, PD1 and CD95 (A) and CD45RA and CCR7 (B) after 5 days of culture in presence of the cytokines when compared to the control condition. Each dot corresponds to one sample. Lines are medians. (C) Heatmap representing the fold-change in all the markers analyzed in our study. (D) Plot showing the principal component analyses comparing the different culture conditions (each symbol represents CD4+ T cells from one individual) and the contribution of the variables analyzed.

**Figure 2. Contrasting effects of IL-2, IL-7 and IL-15 vs IL-21 on the metabolic activity of CD4+ T cells.** Relative changes in basal and maximal glycolysis (A) and basal and maximal respiratory capacity (OXPHOS) (B) after 5 days of culture in presence of the cytokines when compared to the control condition. (C) Ratio of maximal glycolysis and maximal OXPHOS in IL-2 and IL-7 conditions. Each dot corresponds to one sample. Lines are medians (D) Heatmap displaying genes that are differentially expressed in IL-7, IL-10 and IL-21 compared to the unstimulated condition. Each column represents results for CD4+ T cells from one individual.

**Figure 3. IL-2, IL-7 and IL-15 enhanced while IL-21 and IFN- $\alpha$  decreased HIV-1 infection of CD4+ T cells.** (Top) Representative example of the frequency of HIV-1 infection observed in the different culture condition for CD4+ T cells from one donor. (Bottom) Relative frequency of infected cells treated with different cytokines vs the non-treated condition. Each dot corresponds to one sample. Lines are medians.

**Figure 4. Impact of IL-10 and IFN- $\alpha$  counteracted the metabolic enhancing effects of IL-2 and IL-7.** Relative metabolic activities (vs non-treated condition) in cells treated with IL-2 in the absence or presence of IFN- $\alpha$  (A) or IL-10 (C) Relative metabolic activities (vs non-treated condition) in cells treated with IL-7 in the absence or presence of IFN- $\alpha$  (B) or IL-10 (D). (E) Relative infection levels of cells treated IL-2 (left) and IL-7 (right) in the absence or presence of IL-10. Each dot corresponds to one sample. Lines are medians.

## Supplementary Figure Legends

**Figure S1. Impact of IL-2, IL-7, IL-10, IL-15, IL-16 and IFN- $\alpha$  on the size, granularity, CD3, CD4 and CD27 surface markers of CD4+ T cells.** Relative changes after 5 days of culture in presence of the cytokines when compared to the control condition. Each dot corresponds to one sample. Lines are medians.

**Figure S2. Gating strategy for CD4+ T cell subset distribution and the corresponding frequencies of each population.** Reported values are the fold-change in the contribution of each subpopulation to the total pool of CD4+ T cells for each culture condition compared to the non-stimulated condition. Each dot corresponds to one sample. Bars are medians.

**Figure S3. Expression of cytokine receptors in non-stimulated CD4+ T cells.** Proportion of CD4+ T cells expressing CD25 (IL-2Ra), CD122 (IL-2Rb), CD127 (IL-7Ra), CD215 (IL-15Ra). Each dot corresponds to one sample. Lines are medians.

**Figure S4. OCR and PER in non-stimulated and cytokine-treated CD4+ T cells.** The Seahorse XF Analyzer directly measures oxygen consumption rate (OCR) and proton efflux rate (PER). **(A)** Mitochondrial stress test. **(Top)** Schematic representation of the mitochondrial stress test. OCR is measured (in triplicates) before and after the addition of inhibitors to derive several parameters of mitochondrial respiration (relevant here are basal and maximal respiration). **(Bottom)** OCR measured in CD4+ T cells left either non-stimulated or cultured in the presence of the different cytokines. **(B)** Glycolytic rate assay. **(Top)** Schematic representation of the glycolytic rate assay. PER is measured (in triplicates) before and after the addition of inhibitors to derive several parameters of glycolytic capacity of the cell (relevant here are basal and compensatory glycolysis). **(Bottom)** PER measured in CD4+ T cells left either non-stimulated or cultured in the presence of the different cytokines.

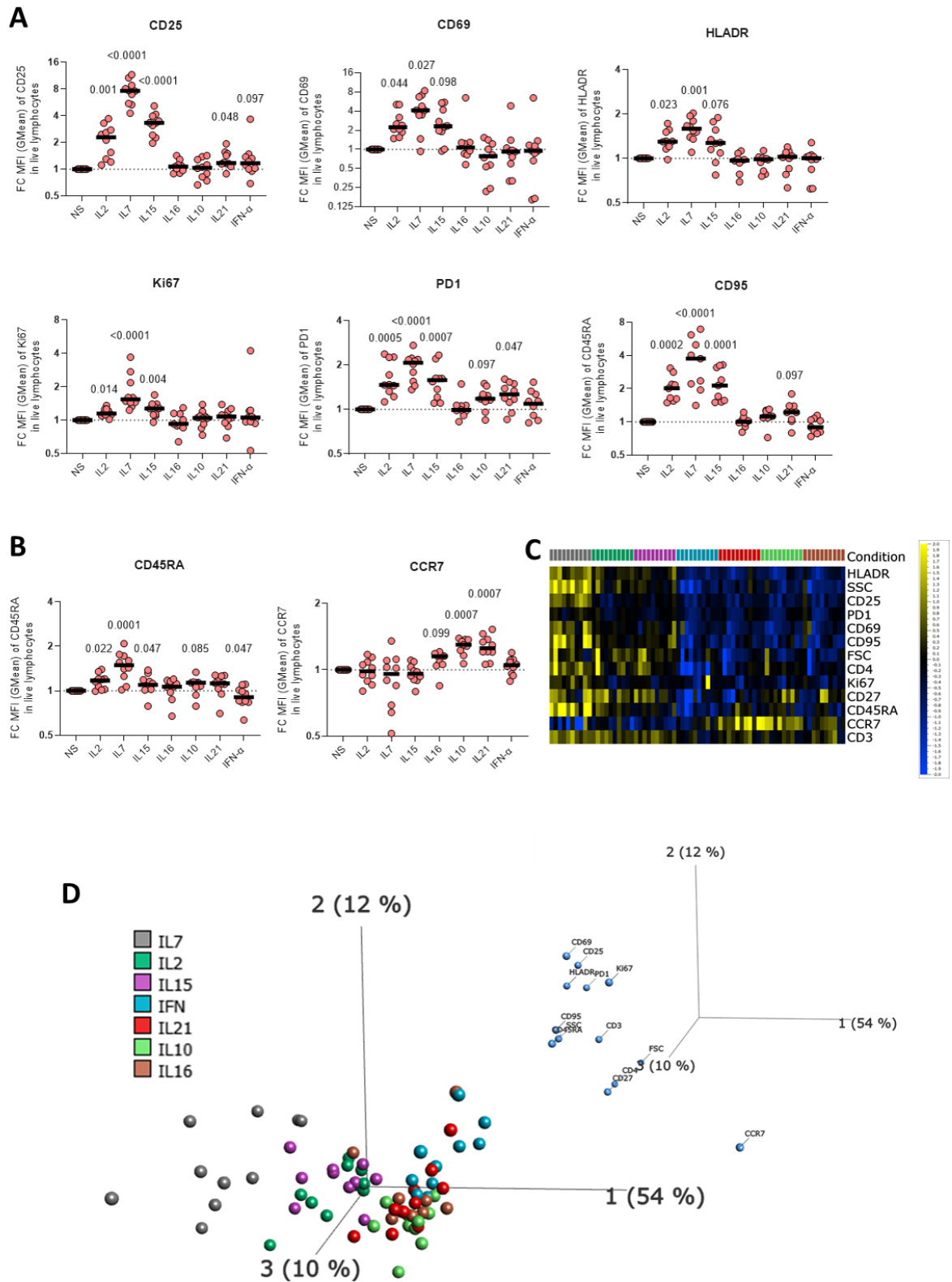
**Figure S5. Ranking of studied genes after analysis with RefFinder (Related to Methods, Figures 1C and 2D).** Ranking of housekeeping gene candidates after analysis with the RefFinder algorithm<sup>891</sup>. IPO8 was the most stable gene present in our panel and was used to normalize our qPCR data.

## Table S1. (Related to Methods, Figures 1C and 2D)

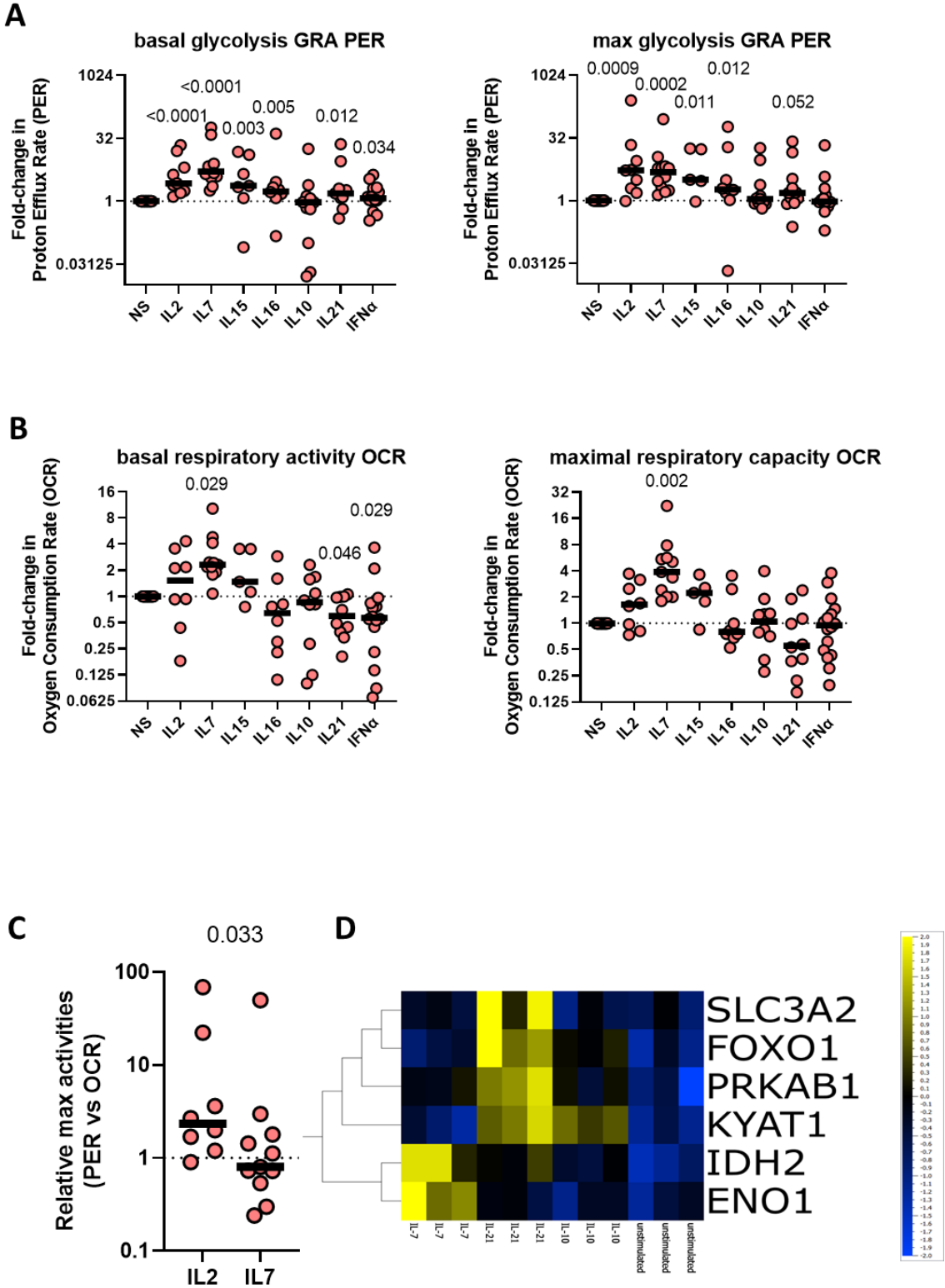
Gene symbols, RefSeq, full gene names and primers used in the transcriptomic q-PCR analysis.



**Figure 1. IL-2, IL-7 and IL-15 increase cellular activation and differentiation of CD4+ T cells.**

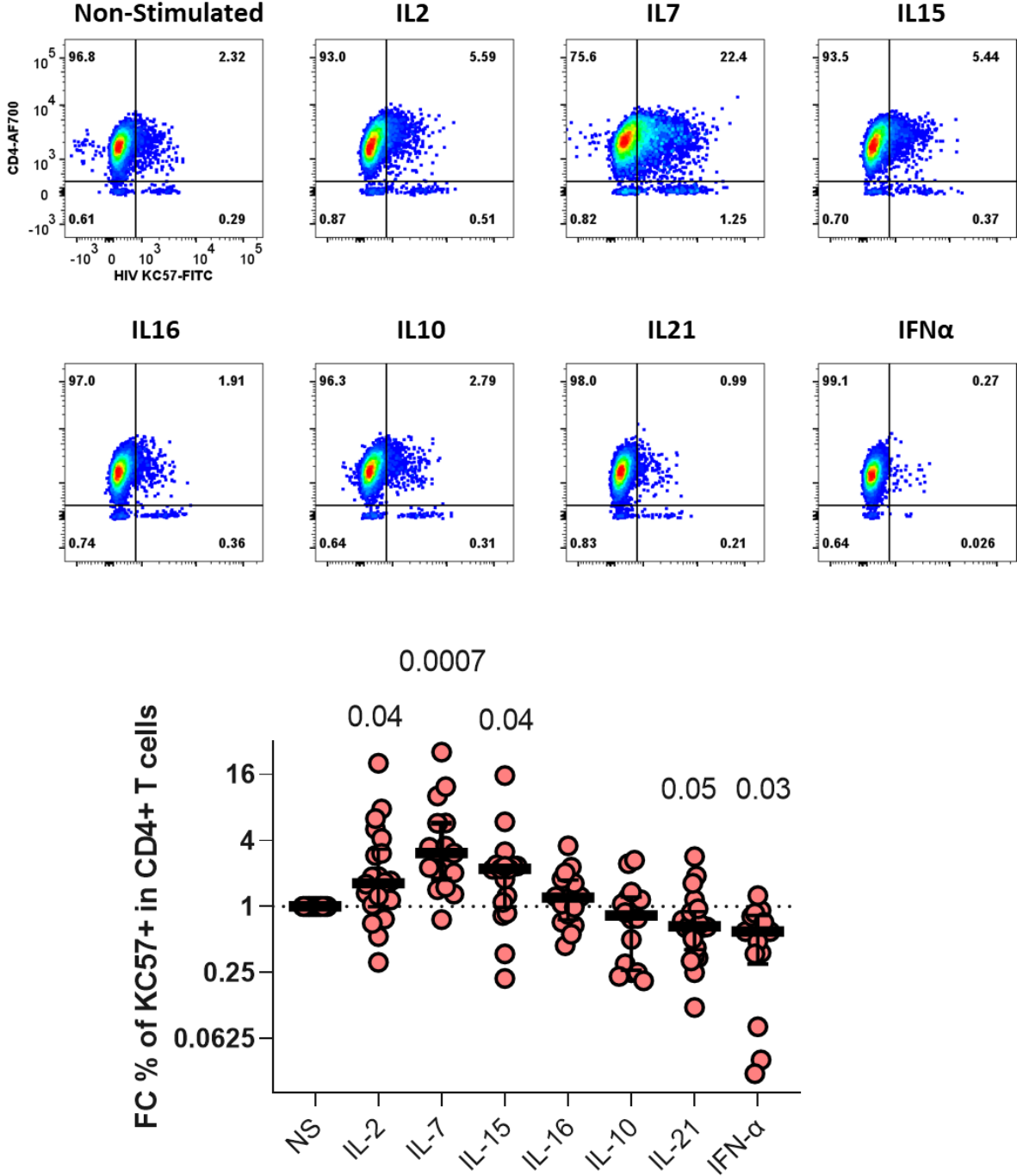


**Figure 2. Contrasting effects of IL-2, IL-7 and IL-15 vs IL-21 on the metabolic activity of CD4+ T cells.**



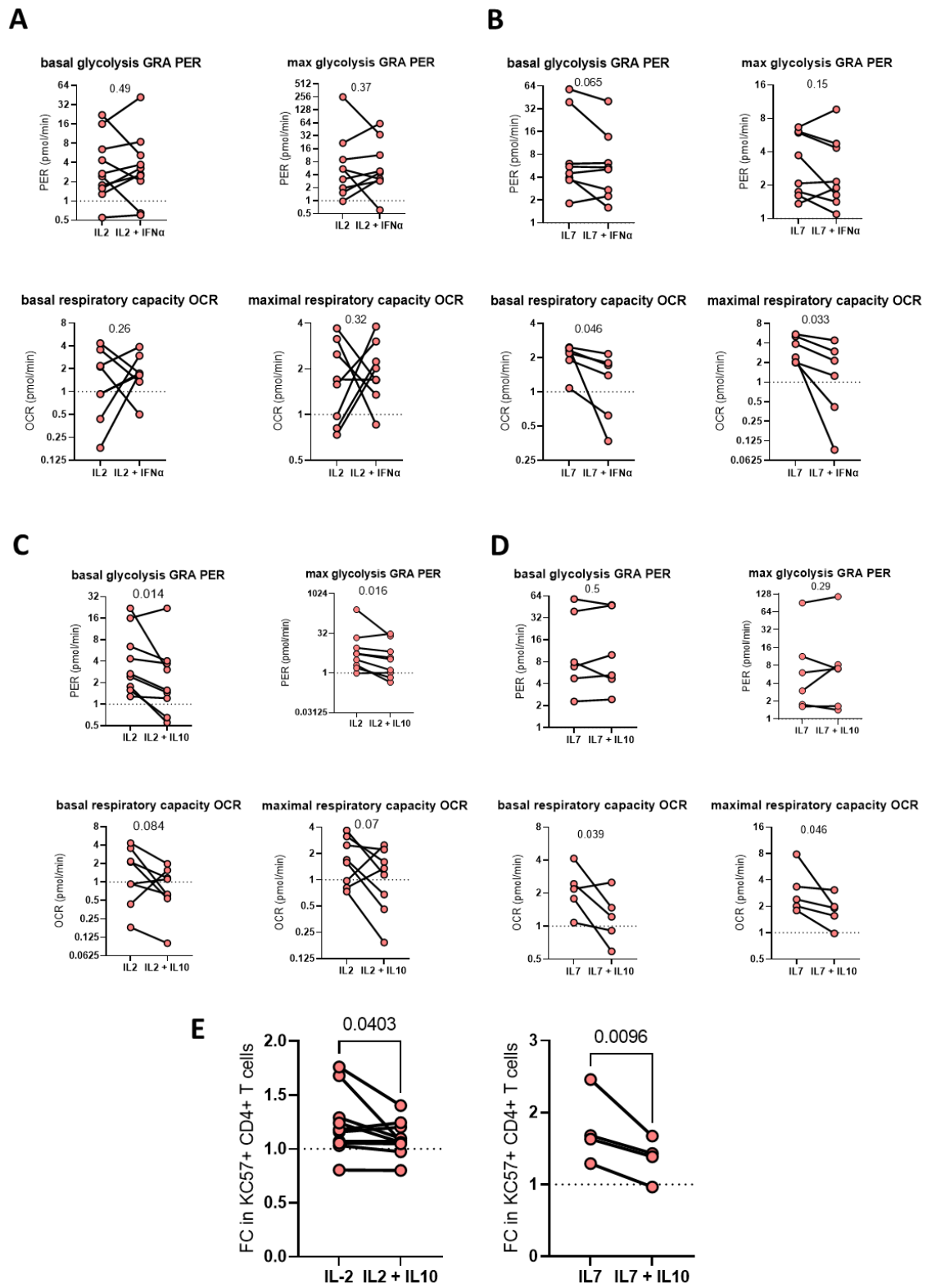
**Figure 2**

**Figure 3. IL-2, IL-7 and IL-15 enhanced while IL-21 and IFN- $\alpha$  decreased HIV-1 infection of CD4+ T cells.**



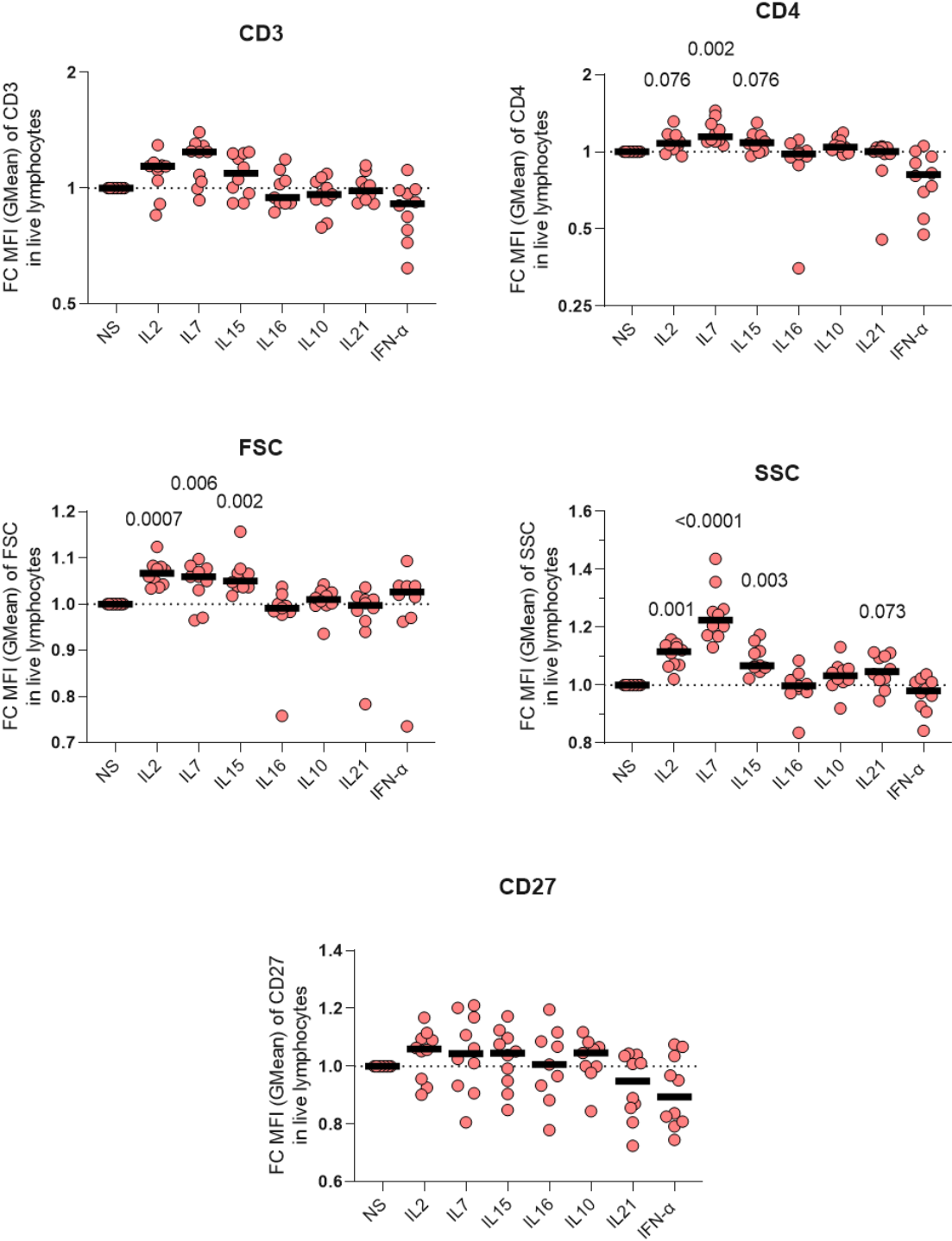
**Figure 3**

**Figure 4. Impact of IL-10 and IFN- $\alpha$  counteracted the metabolic enhancing effects of IL-2 and IL-7.**



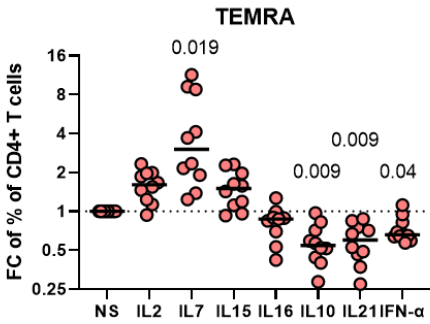
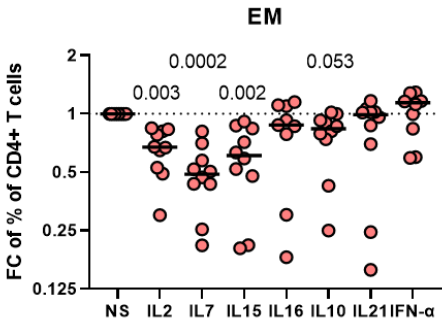
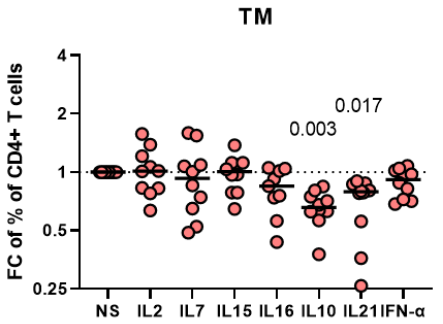
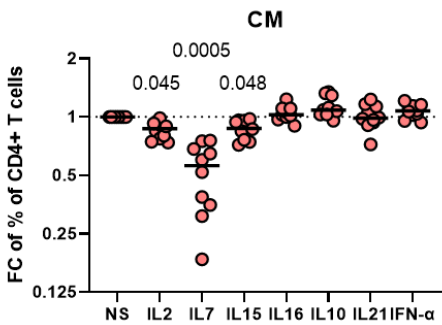
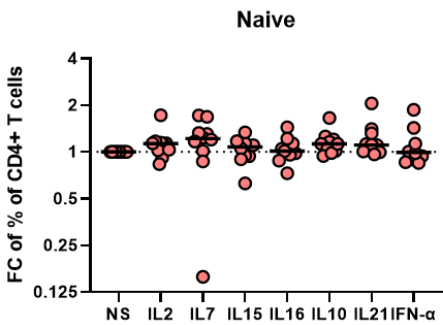
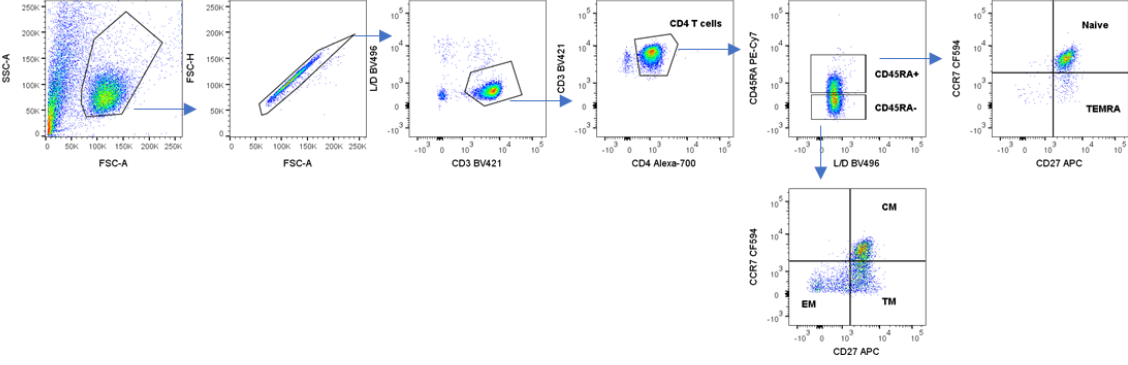
**Figure 4**

**Figure S1. Impact of IL-2, IL-7, IL-10, IL-15, IL-16 and IFN- $\alpha$  on the size, granularity, CD3, CD4 and CD27 surface markers of CD4+ T cells.**



**Figure S1**

**Figure S2. Gating strategy for CD4+ T cell subset distribution and the corresponding frequencies of each population.**



**Figure S2**

Figure S3. Expression of cytokine receptors in non-stimulated CD4+ T cells.

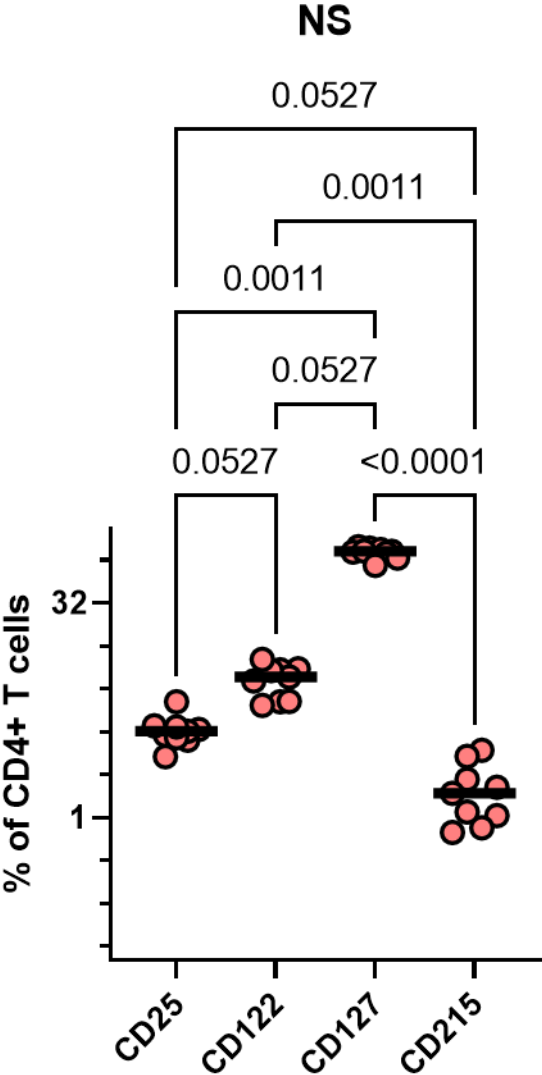
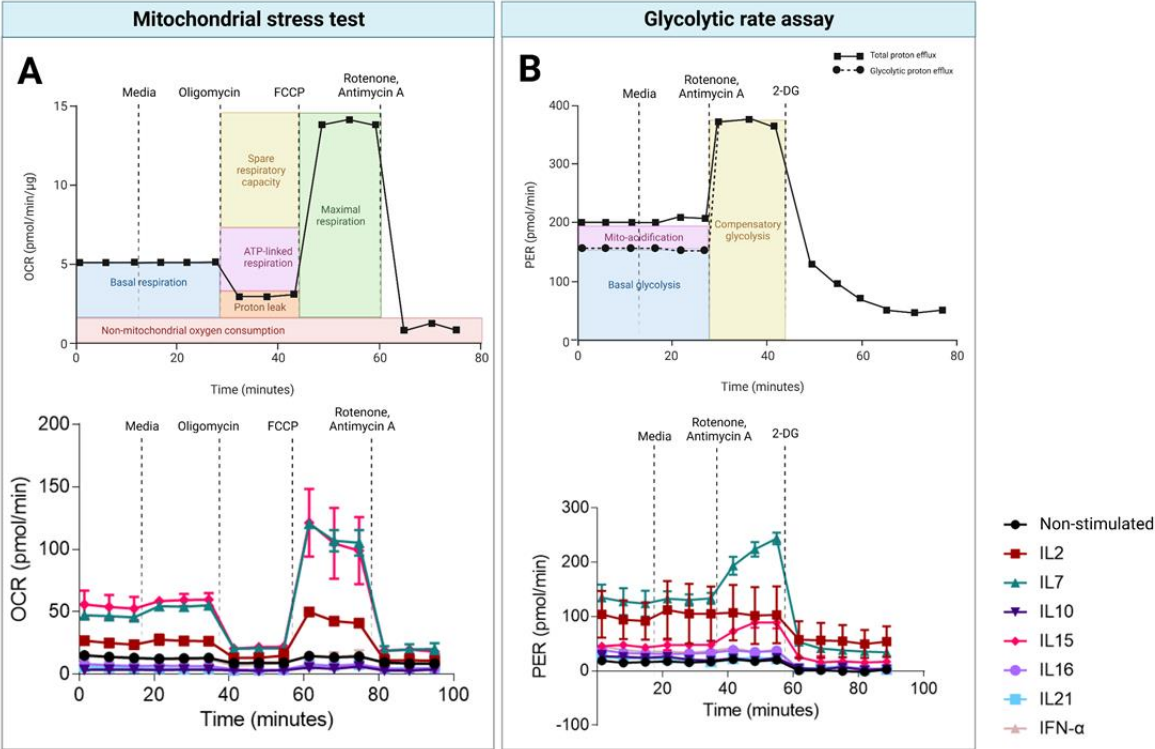


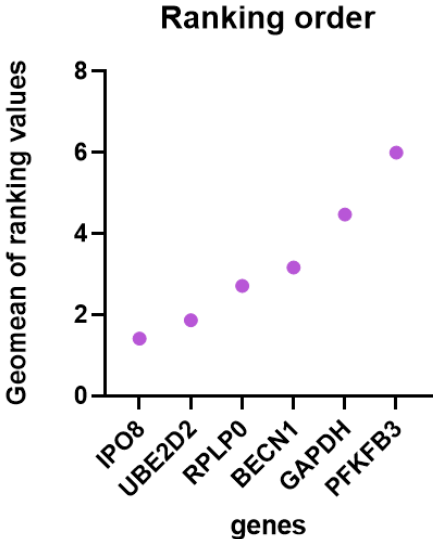
Figure S3

**Figure S4. OCR and PER in non-stimulated and cytokine-treated CD4+ T cells.**



**Figure S4**

**Figure S5. Ranking of housekeeping genes after analysis with RefFinder.**



**Figure S5**



**Table S1. Gene symbols, RefSeq, full gene names and primers used in the transcriptomic q-PCR analysis**

Gene	Design RefSeq	FP	RP	Gene Full Name
ACACA	NM_198839.N	ATCCACAGCTGATCCAGCAA	GCAGAACTGGGAAACCAACC	acetyl-CoA carboxylase alpha
ACADM	NM_001286043.N	GCTACCAAGTATGCCCTGGAA	CATTGCCATTTACAGCAAGATA	acyl-CoA dehydrogenase medium chain
ACADVL	NM_001270448.N	GCACAAAGGCCCAAGAAAGAAA	GAGGGCTCGGTAGACAGAAA	acyl-CoA dehydrogenase very long chain
ACAT1	NM_000019.N	TTCTCATGACGGCAGATGCA	TCTACAGCAGCGTACAGAAA	acetyl-CoA acetyltransferase 1
ACAT2	NM_001303253.N	GTTCAGGAGGGCATGGAAA	CAGTCAATGGCACTCCACCTA	acetyl-CoA acetyltransferase 2
ACLY	NM_001303274.N	CAACCGTCCCATGGACTA	TGCGAGATGCTGGTCATGAA	ATP citrate lyase
ACOD1	NM_001258406.N	TGAAGTCAAGGCCGATTAC	AGCATCCCAAGGTTCTCTA	aconitate decarboxylase 1
ACSS1	NM_001252675.N	AGGCAGGCTACTGCTCTA	CCACACAGCCAAAGATGTCTAC	acyl-CoA synthetase short chain family member 1
ACSS2	NM_001076552.N	TCACTGGCAGGATGATGACA	TTCCACAAGTGTGACTCCA	acyl-CoA synthetase short chain family member 2
AHR	NM_001621.N	TAGGCTACAGCTCACTTAC	TGGCTCCGTTCTTTCAGTA	aryl hydrocarbon receptor
ARG1	NM_001244438.N	TGGCAAGGTGATGGAAAGAAA	CCAGTCCGTCAACATCAA AAC	arginase 1
ARG2	NM_001172.N	AGCTGTGTGATGCTCA	GGCATGGCACTAATGTGAC	arginase 2
ATP5F1A	NM_001001935.N	ACATGGGCTGAGGAATGTTCA	GTCAGGTTCCAAGTCAAAGGAC	ATP synthase F1 subunit alpha
BCAT2	NM_001284325.N	TTTGCTACTGGACCCAGGAA	GACCATCCAGCAGGATAA	branched chain amino acid transaminase 2
BECN1	NM_003766.N	GAGCCAGAGGAAAGACTA	AATCTCGAGACACCATCC	bedin 1
CD36	NM_001001548.N	CTATATTGTGCTTCTTTGGCTTA	TCCAATTTCTGATCTTGAACA	CD36 molecule
CD3D	NM_000732.N	CGTTTCTCTGGCCTGGTA	CTCTACCATGTGATGCTGGTA	CD3d molecule
CD3E	NM_000733.3	GCAACCAAGAGATGCGAAC	CATCACATCCATCTCAAGCA	CD3e molecule
CD3G	NM_000073.N	GCCCAATGACCAAGCTCTA	TTCTCTCAACTGGTTTTCC	CD3g molecule
CD4	NM_001195014.N	AAAGTGTGATCAGGAAGTGAACC	CCCACTCAACAGTCAA	CD4 molecule
CPT1A	NM_001876.N	TCCATGCCATCTCTGTTTACA	AGTGGAACTGTGGATCCAAA	carnitine palmitoyltransferase 1A
CPT2	NM_000098.N	GGTGTCTGCGCAAAACAGAA	GAGTCTCGAGCAGATAGTACA	carnitine palmitoyltransferase 2
CYCS	NM_018947.N	AAGACTGGCCAAATCTCCA	TTGGCGGCTGTGTAAGGATA	cytochrome c, somatic
DNM1L	NM_001278464.N	GCAGGAAGAAAGCACTGATA	AGATGAGTCTCCCGGATTTCA	dynamin 1 like
ENO1	NM_001201483.N	TACCAGCAGTCTGCTGAC	TGAGAACCGCATTGATGACA	enolase 1
FASN	NM_004104.N	GGAGGGGACAGTGCATCAA	GTTTCACTCTCCAGGACAA	fatty acid synthase
FBP1	NM_001127628.N	TTGCTGTGTCCTGTTGGAA	GGTTGCAGAGCATCTTCTCA	fructose-bisphosphatase 1
FOXO1	NM_002015.N	GGTGTCAGGCTGAGGTTA	TTCTCTCAATCTGCTGTGCA	forkhead box O1
G6PD	NM_000402.N	GCCCTCAACGAAGAACATCA	CTCCCGAAGGCTCTTCC	glucose-6-phosphate dehydrogenase
GAPDH	NM_001289746.N	GAGACGGGAACTGTTCTATCA	TGGACTCCACGAGCTACTCA	glyceraldehyde-3-phosphate dehydrogenase
GLS	NM_014905.N	AAGGCAAGCAATGTTGTA	CACTGGCTGATCCAGATCAC	glutaminase
GLUD1	NM_001318905.N	CCAAAGCTGACAACTCTCC	TGTCCTCTCCAGCATCA	glutamate dehydrogenase 1
GOT1	NM_002079.N	CCAAGTGGATTGACCCAAC	TCAAAGAGGGGAAAGAGAAC	glutamic-oxaloacetic transaminase 1
GOT2	NM_002080.N	TCCGGAACAGTGGAAAGGAA	CCTTGTAGGCCATGTCAA	glutamic-oxaloacetic transaminase 2
GPI	NM_001289789.N	ATACGGGAAAGGCTGTGATCA	TCCGTGAAATTTCCCTCA	glucose-6-phosphate isomerase
GSS	NM_001322495.N	AAAAGGGTCTCTGGACCA	GTAGCCATCCCGGAAAGTAAAC	glutathione synthetase
HADHA	NM_000182.N	TTGCGCCATGATGTCTGAA	CGTTGTGTCAGGGAAATCCA	hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha
HK1	NM_001323365.N	TCAATTTCTGCGCAGCAAC	CGCATGCTGTGCTTAAAAACC	hexokinase 1
IDH1	NM_001282387.N	GCTTACTGCGGCTGTAA	ATCATGCCGAGAGAGCCATA	isocitrate dehydrogenase (NADP(+)) 1
IDH2	NM_001290114.N	GAGATGGGAGCCATGACAA	TGTTCCAGGAAAGTCTGTTCA	isocitrate dehydrogenase (NADP(+)) 2
IDO1	NM_002164.N	GGATGCATCACTGGCATA	TTGGCAGTAAGGAAAGCAGCA	indoleamine 2,3-dioxygenase 1
IPO8	NM_006390.N	TTGATGTCAGAAAGGAGGGGAA	ACCCCTCGAGTTAATCTTCCA	importin 8
KEAP1	NM_203500.N	TGGCGCAATGATCACAAGCA	GCATAGATACAGTTGTGCGAGAC	kelch like ECH associated protein 1
KYAT1	NR_148224.N	GCTAGAGGGATGCACTACA	TTCAAGCAGTCACTGCTCA	kyurenine aminotransferase 1
KYAT3	NM_001349450.N	AAGATGGCTGGAGCAACA	GGATCTAATGTCAGTCAAGACTA	kyurenine aminotransferase 3
LDHA	NM_001165414.N	GTGGTCTCTGGGAAACA	TCAAGAGACACCAAGCAACA	lactate dehydrogenase A
LDHB	NM_001174097.N	CAGGATCTCCCGTGTCAACA	ATCCCCGGCATTGAGGATA	lactate dehydrogenase B
LIPA	NM_001288979.N	TGCTGGAACTTCTGTGCAA	TCCCAAGTCAAAGGCTTGA	lipase A, lysosomal acid type
MFN2	NM_001127660.N	ATCAGTCACTGGCTCCAA	GACGTCACTGTGTCAGCA	mitofusin 2
MPC1	NR_073093.N	CATGCCAGCAAAATGAA	TGCAGATGCCGTTTGTAGCA	mitochondrial pyruvate carrier 1
MPC2	NM_001143674.N	GGCCAGCACTGCAAGAA	TGGAATAATACAAGTGAAGTCTGAC	mitochondrial pyruvate carrier 2
NDUFB8	NM_001284367.N	CCTCAGCATGAGAGACTCA	TCCAACGGTCTCTGTTGTA	NADH:ubiquinone oxidoreductase subunit B8
NFE2L2	NM_001145412.N	GACGAAGTTGGGAGGAGCTA	GGTATGCTCAACCAAGTGTGCA	nuclear factor, erythroid 2 like 2
NOS2	NM_000625.4	CTCACAGCTTGGACCTCA	GAGATTTGAGCTCAATGGTGAAC	nitric oxide synthase 2
NR4A2	NM_006186.N	TGGCTGTTGGATGGTCAA	TTCTCGTTTCSAGGGGCAA	nuclear receptor subfamily 4 group A member 2
NR1	NM_001293163.N	CGCAGAGAGAGCAAAAGCA	CCCGCCATGCTGTTTATAAC	nuclear respiratory factor 1
OGDH	NM_001363523.N	CTAGTGAAGTGGGGTGGACTA	CCAGCTCTTCTGATGACA	oxoglutarate dehydrogenase
OPA1	NM_130837.N	CCAAGTGAAGCTGGCAGAAA	TCAATGGGAAAGCTTCTTCA	OPA1 mitochondrial dynamin like GTPase
PDHA1	NM_001173454.N	GGTGTGCTGCTGCTAATGTA	TGTGCTGGTGAACGCTGAA	pyruvate dehydrogenase E1 subunit alpha 1
PDHB	NR_033384.N	AGCTGCAGCAGTGTACTTAA	TGGTTCATGTCAATGGTCTA	pyruvate dehydrogenase E1 subunit beta
PKD1	NM_001278549.N	ACCAAAGCTGGTGTGAGAC	AAGAGGTGATGGGCAATCCA	pyruvate dehydrogenase kinase 1
PKD2	NM_002611.N	GGACAAAGATCCCGAGGAC	GTGTTGTGCGGCTTCC	pyruvate dehydrogenase kinase 2
PKD3	NM_00142386.N	AGGGTACCTGCTGTAA	ACACCACCACTAGGTCA	pyruvate dehydrogenase kinase 3
PFKFB3	NM_001323017.N	CGGGACAGGACAGTACTA	TCCAATGACTGCTCCAA	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3
PFKL	NM_001002021.N	ACGGAGAGATGAAAGCAGACA	CAGGAACTCGTGGTGTAGTA	phosphofruktokinase, liver type
PGD	NM_001304452.N	CTCATGCCAGGAGGAAACA	TTTTGCAAGTGCCTGGAA	phosphogluconate dehydrogenase
PGK1	NM_000291.3	GTGGAATGGCTTTTACCTTCC	CTTGCTCTCTTCACTCA	phosphoglycerate kinase 1
PGM2	NM_018290.N	TAAAGCCCGGATGCTGATA	CCCCAATCTATTGCTGAAA	phosphoglucomutase 2
PKM	NM_182470.N	CGGGTGAATTTGCCATGAA	CATCCGCTCAGCAAAATGAC	pyruvate kinase M1/2
PNPLA2	NM_020376.N	ACCTCTACCGCTCTCCAA	CAGGCCATCCCGTATCC	patatin like phospholipase domain containing 2
PPARA	NM_001362873.N	CGGGTCACTCTCAAGAA	AGCCATACAGAGTCTCCA	peroxisome proliferator activated receptor alpha
PPARG	NM_001354668.N	CAGATCCAGTGTGAGAT	GAGATGCAGGCTCCACTTT	peroxisome proliferator activated receptor gamma
PPARGC1A	NR_148983.N	TTCTCCCACTTCCCTATCC	CTGTAGCAAGTTCGCTCA	PPARG coactivator 1 alpha
PRKAA2	NM_006252.N	ACCTGGTTGATAACAAGGACTA	ACAGATCTGCTCCACTAC	protein kinase AMP-activated catalytic subunit alpha 2
PRKAB1	NM_006253.N	GGGTCTTCAACAATGAGGAGTA	GCAGATCCAGGATGCTCAA	protein kinase AMP-activated non-catalytic subunit beta 1
RPLP0	NM_053275.N	GGCACTGGAAATGCTCAA	CACATTTGCTGCTCCACAA	ribosomal protein lateral stalk subunit P0
RPS6	NM_001010.N	AGAAAGCTATGGCCACAGAA	ACCACTGATGGCCACATA	ribosomal protein S6
SCAP	NM_012235.N	ACCAAAGTGTGCTCAAC	GACCACTCTGCTGCTTA	SREBF chaperone
SDHA	NM_004168.N	GCCAGGACCTAGAGTTGTCA	CTCCACAGCATCTTCCGTAA	succinate dehydrogenase complex flavoprotein subunit A
SLC16A1	NM_001166496.N	TTCTCCATATGTTGGCTGTCA	GACAAGCAGCCCAACAA	solute carrier family 16 member 1
SLC16A4	NM_004696.N	CTGCTCTTGGACCACTA	AGAAGCAATGATGTTGGGTA	solute carrier family 16 member 4
SLC22A5	NM_001308122.N	CAATGGGATGTTGTGCTTCC	GGGACTGCTGCTTGGGAA	solute carrier family 22 member 5
SLC25A1	NM_001256534.N	TGTGCGCACTGGAGACC	CCGTTGGAAAGATCTCTGTA	solute carrier family 25 member 1
SLC25A20	NM_000387.N	TGGAGAACGGATCAAGTGTCTA	TTGCACATCCAGTCAACA	solute carrier family 25 member 20
SLC3A2	NM_001012662.N	TGGCTGATGGGCAAAATATCAC	GCTGAAGTCCGAGGAGTGA	solute carrier family 3 member 2
SLC7A5	NM_003486.N	TTGGGGTCTGGTGGAAA	CCTGATGAGCTCTGACAC	solute carrier family 7 member 5
SOAT1	NM_001252511.N	CAGCTTCCCGTCACTATA	GGCACTTCTCTGACAAA	sterol O-acyltransferase 1
SOD2	NM_001322819.N	CAACCGCAGATCATGCA	CTTCTCTGGTGCAGTCA	superoxide dismutase 2
SREBF1	NM_001005291.N	CAGCAACAGAACTCAAGCA	CGCGACCAAGATCTTCA	sterol regulatory element binding transcription factor 1
TALDO1	NM_006755.N	CATCAACTGGGAGGAAATCA	TGGGGAGAGGAGAAAGTAA	transaldolase 1
TFAM	NR_073073.N	GAAGACTGAAAGGAAAACCTGAA	CATTGTCTTCCAAAGACTCA	transcription factor A, mitochondrial
UBE2D2	NM_181838.1	ACAAGTGGTCTCAGCACTAA	GGATCATCTGGATGGGATCAC	ubiquitin conjugating enzyme E2 D2
VDAC1	NR_036625.N	GGTGGTCTGTGCTAGGTA	ACTGGGAATTTGAGTCTCA	voltage dependent anion channel 1
XBP1	NM_005080.N	CATGGATCTGGCGGATTGAC	TGGTCCAAAGTTCAGAA	X-box binding protein 1

# DISCUSSION

A relatively novel notion in immunology postulates that metabolic activity and immune cell activation are tightly linked. Immunometabolism, the investigation of the cross-talk between bioenergetic pathways and immune cell functions, is becoming recognized as a significant scientific area that holds many promises.

Thus far, immunology has placed a strong emphasis on researching how immune cells receive signals in order to recognize antigens and coordinate the immune response. However, until recently, the majority of research focused on molecular aspects and essentially disregarded any relevance for metabolism in this field. Meanwhile, cancer research had been focusing on metabolism for many years, since the uncontrolled growth of cancer cells revealed targetable metabolic weaknesses. Attention to immunometabolism increased with the identification of a metabolic parallel between the two fields, founded in what became to be known as “Warburg metabolism”, which consists of an increase in the rate of glucose uptake and preferential production of lactate, even in the presence of oxygen, to metabolically support their activation process<sup>896</sup>. The field of immunometabolism has grown explosively since then.

Viral replication and mobilization of an immune response have in common the dependency on the cell’s available nutrients, metabolic intermediates and energy. Typically, HIV-specific CD4<sup>+</sup> T cells are involved in the anti-HIV response and must specifically adjust their cellular metabolism to help in the development of the immune response. Furthermore, because they are the site of viral replication, the virus will reroute a significant portion of CD4<sup>+</sup> T cell machinery and metabolic intermediates, thereby altering cellular metabolism and jeopardizing the immune response. Furthermore, because it supplies the cell with extra metabolic resources, this mobilization of the host cellular metabolism may be favorable to the virus. Distinct CD4<sup>+</sup> T cell subsets use different metabolic pathways to support their function, with TH1, TH2 and TH17 cells engaging glycolysis and TREG cells preferring fatty acid oxidation<sup>326</sup>. Interestingly, HIV preferentially infects certain subsets of CD4<sup>+</sup> T cells versus others that seem to be relatively resistant to infection. Others and we have proposed that the metabolic status of the CD4<sup>+</sup> T cells is a key determinant of their HIV susceptibility.

My thesis work was founded on several previous observations. Among CD4<sup>+</sup> T cell subsets, there is a correspondence between the hierarchy of infection and the hierarchy of cellular metabolism. Subsets that are associated with higher infection rates are characterized by high metabolic activities, including glycolysis and OXPHOS. Even within the same subset, in cells sharing the same activation and differentiation phenotype, if we divide cells based on their metabolic activity, we find that the rate of infection is higher in cells that exhibit relatively high metabolic activity compared to cell who are metabolically quiescent. Susceptibility to HIV has been knowingly attributed to cell cycle progression, and this is explained by the fact that when the cell needs to proliferate or differentiate it upregulates processes and molecules that the virus requires to establish infection. However, cell cycle progression and responding to stimulatory signals are powered by cellular metabolism. Without cellular metabolism there is not enough energy nor blocks to sustain the enhanced activity of the cell. We hypothesized that HIV also depends on this cellular metabolic activity and that if we “revert” the basal metabolic activity of the cell, we would be able to restore an insufficient environment for the virus to replicate.

Because of their high metabolic requirements, infected cells (or cells that are highly susceptible to infection) may also be especially sensitive to suboptimal amounts of molecules that interfere with metabolic processes, which might lead to the specific elimination of these cells without affecting other CD4+ T cells, as has been shown for cancer vs healthy cells.

Our first goal was to test this hypothesis by employing different metabolic inhibitors that would interfere with intrinsic or viral-induced metabolic environments that favor infection. Thanks to research on cancer and metabolic diseases, many drugs that could be repurposed, targeting different metabolic pathways, already exist. Our results suggest that glycolysis, OXPHOS and FAS are metabolic pathways that support infection with replication competent HIV. In particular, we observed a consistent decrease in the frequency of infected cells when infections were done in the presence of two different molecules targeting glycolysis, 2-DG an analog of glucose, and 3-BrPA an allosteric inhibitor of HK-II. We further observed inhibition of infection in the presence of Zaprinast, which is a phosphodiesterase (PDE) inhibitor that was shown to inhibit pyruvate (a downstream product of glycolysis) import to the mitochondria. These results are consistent with previous observations from the team using single cycle VSV-G pseudotype HIV particles and point to a prominent role of glucose metabolism, including feeding mitochondrial respiration. We also obtained consistent inhibition of HIV infection using three molecules that targeted fatty acid synthesis (Orlistat and C75, which target FASN; TOFA, which targets ACC).

Being devoid of any type of cellular machinery and limited by a relatively very small genome, viruses adapt mechanisms to hijack cellular component to promote their propagation. Almost every step in the virus replication cycle involved what became to be known as “molecular hijacking”. In the introduction of this manuscript, under the “host-dependence factors necessary for HIV life cycle” section, we highlighted some of the known examples of the molecular hijacking employed by HIV. In addition to molecular hijacking, viruses are dependent on the cell’s metabolism and they also hijack many metabolic intermediate to achieve their replication cycle. Our results suggest that glycolysis and FAS appear to be very important for HIV infection of CD4+ T cells. High glycolysis may favor early viral replication steps by enhancing the biosynthesis of dNTPs and by providing rapid ATP for the activities of viral enzymes upon cell entry. Aerobic glycolysis also generates the oxidized form of nicotinamide adenine dinucleotide (NAD<sup>+</sup>), a redox carrier involved in many metabolic processes<sup>910</sup>. NAD<sup>+</sup> also serves as a nucleotide analog in DNA ligation and messenger RNA cap synthesis<sup>911,912</sup>. A recent report suggests that aerobic glycolysis is also important for virion infectivity by retaining the glycolytic enzymes and preventing their incorporation into the viral particle<sup>897</sup>. In addition, the conversion of pyruvate into acetyl-CoA in the mitochondria through the TCA cycle favors  $\alpha$ -tubulin acetylation and the stabilization of microtubules co-opted for cytoplasmic transport of HIV<sup>365</sup>. On the other hand, acetyl-CoA contributes to histone acetylation and its generation can modulate HIV transcription<sup>377</sup>. Additionally, acetyl-CoA is a key precursor of de novo cholesterol synthesis and cholesterol has been found to aid HIV infection by forming lipid rafts, which are essential for viral entry, trafficking of HIV proteins and viral particles budding<sup>378</sup>. Moreover, cholesterol constitutes up to 40% of the total lipids in the HIV viral membrane and is necessary for infectivity<sup>379–382</sup>. Acetyl-CoA also contributes to FAS and production of myristic acid. This latter binds HIV Gag precursor, a step that is required for Gag to attach to

membrane rafts, the assembly of the viral capsid, and the release and maturation of viral particles<sup>383</sup>.

Therefore, glycolysis and FAS may be involved in the regulation of different steps of the viral replication cycle. If that is the case, blocking simultaneously both metabolic pathways may have synergistic inhibitory effects on HIV infection. Moreover, the simultaneous targeting of different metabolic pathways may limit compensatory metabolic switches, enhancing the efficacy of the intervention. These questions need to be further explored with the perspective of assessing the potential of therapeutic approaches targeting immunometabolism to block HIV infection and/or selectively eliminate infected cells.

Contrary to the results that we obtained with inhibitors targeting glucose metabolism and FAS, the use of glutaminolysis inhibitors (L-DON and BPTES) did not result in a consistent impairment of HIV infection in our experimental conditions. Clerc *et al.* have shown that the fueling of the TCA cycle with glutamine intermediate  $\alpha$ -KG rescues HIV early steps under conditions of attenuated glutaminolysis, and our group had also previously observed a relative decrease of infection in the presence of L-DON using HIV NL4.3-VSVG-pseudotype virus lacking env and nef<sup>361,364</sup>. We therefore expected that blocking glutaminolysis would have similar effects as glycolysis inhibition on HIV infection. However, this was not our observation. This unexpected outcome might be explained by different reasons. It is possible that L-DON interfered with early steps of HIV infection, as shown in the experiments with single cycle virus in the Clerc *et al.* and our previous studies, but that the effect does not have the same impact on late steps, is transient or diluted with several replication cycles when using a productive virus<sup>361,364</sup>. It is also possible that the interaction with env and nef rewires the cell's metabolism making them less dependent on glutaminolysis. Another explanation is that the cell could more easily switch from a glutaminolysis-based TCA and OXPHOS to a glycolysis-reliant TCA cycle than the opposite. In addition, we used the drugs at nontoxic doses and we aimed for a minimum of 70% viability in our culture, with an average of roughly 90%, which may have precluded revealing a clear effect of the drugs targeting glutamine metabolism. Moreover, in experiments with higher molecule concentrations we observed a relative increase in the cytotoxicity effect of L-DON on infected cells, which suggest an enhanced dependence of infected cells on glutamine metabolism.

We also obtained unexpected results using metformin (MET); a drug used in the treatment of type 2 diabetes that is the subject of drug repurposing for cancer treatment and prevention. MET has been reported to inhibit complex I of the electron transport chain and reduce the incorporation of glucose- and glutamine-dependent carbon into TCA cycle metabolite pool, resulting in an inhibition of both OXPHOS and mitochondrial-dependent biosynthesis<sup>898,899</sup>. However, in our experiments, MET did not result in a decrease in HIV infection, on the contrary we even observed a trend towards an enhancement in viral replication. This could be explained by the hormetic effect that MET can exert based on its concentration<sup>900</sup>. At milimolar concentrations MET inhibit complex I of the electron transport chain and thus inhibit mitochondrial activity; whereas at the micromolar concentration MET activates the mitochondrial electron transport chain, increasing ATP production and limiting oxidative stress. We can hypothesize that in our experimental conditions MET might have exerted a boosting effect on the mitochondrial activity and increase HIV infection. Unfortunately, we did not test for the impact of each metabolic drug on cellular metabolism in our experiments, therefore it is difficult to dissect the exact effect MET had on CD4+ T cells metabolism. We

intend to complete the study evaluating the impact of each drug using extracellular flux analysis (Seahorse).

Our data also suggests that some cytokines (such as IL-2, IL-7 and IL-15) may enhance infection by providing a favorable metabolic environment for viral replication, others (such as IL-10, IL-21 and IFN- $\alpha$ ) may induce unfavorable metabolic conditions for the setting of infection. We chose to focus on these cytokines because their levels are elevated during the acute phase of HIV infection, a period when the seeding of the HIV reservoir occurs, they are known to play a role in T cell homeostasis and have been shown to impact HIV replication *in vitro*. There are various contradictory reports concerning the role of cytokines, particularly IL-2, IL-7, IL-15 and IFN- $\alpha$  in HIV infection *in vivo*. Cytokines are pleiotropic, and the overall effects of a specific cytokine on HIV replication or metabolism frequently reflect a mix of inducing and inhibitory activities. The complexities of the interactions between cytokines and T cells make it impossible to conclude on the relevance of a single component of their regulatory activity on HIV replication *in vivo*; however, some of these interactions may be analyzed under more controlled *in vitro* circumstances.

We observed that cytokines that provoked a significant increase in glycolysis and OXPHOS were associated to an increase in HIV infection. Whereas cytokines that induced a decrease in OXPHOS, such as IL-21 and IFN- $\alpha$ , resulted in an inhibition of HIV infection. IL-10 did not result in a significant impact on cellular metabolism nor HIV infection on its own under our experimental conditions, when it was combined with IL-2 or IL-7 it counteracted the cytokines-induced increase in cellular metabolism and decreased the susceptibility of CD4+ T cells to HIV infection. Although cytokines, and especially IFN- $\alpha$ , may alter the expression of numerous antiviral molecules, their influence on CD4+ T cell metabolism was analogous to their capacity to enhance or inhibit HIV replication. Our findings that metabolic inhibitors can modulate HIV infection and other reports demonstrating a role for cytokines in metabolic reprogramming suggest that metabolic rewiring can be one of the ways cytokines influence susceptibility to HIV infection<sup>901–905</sup>. It will be interesting to explore the changes in the metabolic status of CD4+ T cells that may occur in PLWH during acute and chronic infection and how these differences may impact the seeding of the viral reservoir and the survival program of infected cells.

Our results show that three metabolic pathways glycolysis, OXPHOS and fatty acid synthesis are crucial for HIV infection. Their suboptimal inhibition can prove efficient *in vivo* as it may allow for potent blockade of HIV infection with minimal toxicity and side effects. We previously showed that suboptimal inhibition of glycolysis with 2-DG causes the death of infected CD4+ and hinders HIV spread from CD4+ T cell reservoirs. In other studies, by supplying CD4+ T cells with the poor glycolysis substrate galactose, virion release in the culture supernatant was decreased to between 20% and 60% of the quantity generated in glucose-containing cultures<sup>375</sup>. Similar interventions coupled to other metabolic drugs that are shown to inhibit HIV infection, or to other curative strategies such as the “*shock and kill*” approach for example, could prove extremely potent in eradicating the HIV reservoir. Metabolic drugs combined to ART can also present an opportunity to increasing ART efficiency. Similar approaches are employed in cancer where pyrimidine nucleotide metabolism is targeted to increase sensitivity to chemotherapy<sup>906</sup>. Nevertheless, it is important to consider a possible hierarchy of metabolic routes that are important for a sustained infection, and that the virus may redirect cellular metabolism toward the second option when the first is unavailable. Therefore,

strategies that target more than one metabolic pathway may lead to better results. Finally, metabolic inhibitors can be used to restore a “normal” metabolic activity of CD4+ T cells. This could “revert” the intracellular environment of the cell from susceptible to resistant, which could lead for elimination of reservoir cells due to their inability to sustain the metabolic requirements of the integrated virus. Given that cytokines are being investigated for the purpose of HIV targeted immunotherapies, it may be important to consider the impact of an individual cytokine on viral replication before implementing it into a therapeutic approach. On the other hand, by evaluating the influence of cytokines on cellular metabolism, we could identify candidate cytokines for HIV therapy. Moreover, combination between cytokines and metabolic inhibitors could also be useful. Blocking glycolysis or OXPHOS in IL-7 treated cells may still allow for beneficial effects of IL-7 without the increase in metabolic activity that would enhance viral replication. Similarly, reinforcing IFN- $\alpha$  or IL-21-induced decrease in OXPHOS could enhance the antiviral function of these cytokines. As a result, lactate dehydrogenase inhibition was discovered to work in conjunction with IL-21 to increase CD8+ T cell stemness and antitumor immunity<sup>779</sup>.

Another point to keep in mind is the consideration of such interventions depending on the phase of infection. For example, glycolysis may be of importance for HIV during the early stages of infection because it allows for its rapid entry and integration into the host cell genome. However, high glycolytic activity during the late stages may compromise the latency of the virus by acting on its transcription and therefore its detection by the immune system. Glycolysis at this stage may be also beneficial for the host immune cells for them to generate enough biomass and energy for the mounting of an immune response. For instance, influenza was found to also increase and benefit from glycolysis during the early stages of infection<sup>907</sup>. However, during the late stages, the virus was shown to inhibit glycolysis by blocking the glucose-6-phosphate isomerase (GPI) reaction<sup>908</sup>. MET supplementation of ART has been shown to be beneficial in non-diabetic PLWH<sup>369</sup>. In these settings, MET was shown to attenuate residual HIV transcription and control inflammation by improving glucose metabolism and by regulating intracellular immunometabolic checkpoints such as AMPK and mTOR, in association with microbiota modification. However, our *in vitro* experiments show that MET increased HIV replication in some of our samples. It is therefore plausible that the benefit of MET *in vivo* relies more on its enhancement of the function of cytolytic components of the immune system such as NK and CD8+ T cells, as has been also suggested for the metabolic reprogramming of HIV-specific CD8+ T cells using IL-15 or GSK3 inhibitors<sup>399,909</sup>. The fine tuning of these activities may make therapies targeting immunometabolism an integrated approach promoting the enhancement of the antiviral activities of immune effectors in a context where the infection and/or survival of target cells is disadvantaged.

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# APPENDICES

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*Mikhailova A, Valle-Casuso JC, David A, Monceaux V, Volant S, Passaes C, Elfidha A, Müller-Trutwin M, Poyet JL, Sáez-Ciri3n A. Antiapoptotic Clone 11-Derived Peptides Induce In Vitro Death of CD4<sup>+</sup> T Cells Susceptible to HIV-1 Infection. J Virol. 2020 Jul 1;94(14):e00611-20. doi: [10.1128/JVI.00611-20](https://doi.org/10.1128/JVI.00611-20). PMID: 32350074; PMCID: [PMC7343195](https://pubmed.ncbi.nlm.nih.gov/PMC7343195/)*



## Antiapoptotic Clone 11-Derived Peptides Induce *In Vitro* Death of CD4<sup>+</sup> T Cells Susceptible to HIV-1 Infection

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**ABSTRACT** HIV-1 successfully establishes long-term infection in its target cells despite viral cytotoxic effects. We have recently shown that cell metabolism is an important factor driving CD4<sup>+</sup> T cell susceptibility to HIV-1 and the survival of infected cells. We show here that expression of antiapoptotic clone 11 (AAC-11), an antiapoptotic factor upregulated in many cancers, increased with progressive CD4<sup>+</sup> T cell memory differentiation in association with the expression of cell cycle, activation, and metabolism genes and was correlated with susceptibility to HIV-1 infection. Synthetic peptides based on the LZ domain sequence of AAC-11, responsible for its interaction with molecular partners, were previously shown to be cytotoxic to cancer cells. Here, we observed that these peptides also blocked HIV-1 infection by inducing the death of HIV-1-susceptible primary CD4<sup>+</sup> T cells across all T cell subsets. The peptides targeted metabolically active cells and had the greatest effect on effector and transitional CD4<sup>+</sup> T cell memory subsets. Our results suggest that the AAC-11 survival pathway is potentially involved in the survival of HIV-1-infectible cells and provide proof of principle that some cellular characteristics can be targeted to eliminate the cells offering the best conditions to sustain HIV-1 replication.

**IMPORTANCE** Although antiretroviral treatment efficiently blocks HIV multiplication, it cannot eliminate cells already carrying integrated proviruses. In the search for an HIV cure, the identification of new potential targets to selectively eliminate infected cells is of the outmost importance. We show here that peptides derived from antiapoptotic clone 11 (AAC-11), whose expression levels correlated with susceptibility to HIV-1 infection of CD4<sup>+</sup> T cells, induced cytotoxicity in CD4<sup>+</sup> T cells showing the highest levels of activation and metabolic activity, conditions known to favor HIV-1 infection. Accordingly, CD4<sup>+</sup> T cells that survived the cytotoxic action of the AAC-11 peptides were resistant to HIV-1 replication. Our results identify a new potential molecular pathway to target HIV-1 infection.

**KEYWORDS** AAC-11, CD4 T cells, apoptosis, cell metabolism, human immunodeficiency virus, susceptibility to infection

**H**uman immunodeficiency virus type 1 (HIV-1) disease is a persistent viral infection that has claimed millions of lives around the globe. The era of combination antiretroviral therapy (cART) has extended the life expectancy of people living with HIV and improved their quality of life. However, although cART blocks viral replication, it does not eliminate infected cells, which persist despite decades of treatment and originate viral rebound if therapy is interrupted (1–3). There is, therefore, a need to identify characteristics of HIV-infected cells that could be potentially targeted by new

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therapeutic strategies. CD4<sup>+</sup> T cells are primary targets of HIV-1, but the cells differ in their relative susceptibility to infection (4–6). HIV requires a particular cell environment providing abundant factors that the virus exploits to sustain its replication cycle. Susceptibility to HIV infection *in vitro* increases with CD4<sup>+</sup> T cell differentiation. Naive CD4<sup>+</sup> T cells are most resistant, while central memory (Cm), transitional memory (Tm), and effector memory (Em) CD4<sup>+</sup> T cells are progressively more susceptible to the virus. We have recently shown that these differences are, at least in part, related to the increased metabolic activity associated with progressive differentiation of these subsets (7). Immunometabolism is a critical element in the regulation of T cell differentiation, survival, and function (8). Upon antigenic stimulation, T cells upregulate metabolic fluxes to provide the energy necessary to support cellular processes and to increase the pool of substrates necessary for building proteins, lipids, nucleic acids, and carbohydrates. This metabolically rich environment is necessary for the establishment of both productive and latent HIV infections (7, 9, 10), as is also the case for other infections (11–13), and may offer new opportunities to tackle HIV.

In a *post hoc* analysis of results obtained in our previous study (7), we found that antiapoptotic clone 11 (AAC-11) (also known as apoptosis inhibitor 5 [API5]) was significantly correlated with infection in different subsets of memory CD4<sup>+</sup> T cells. The antiapoptotic activity of AAC-11 might contribute to the survival of metabolically active cells. Indeed, AAC-11 is overexpressed in many cancers (14) and allows cancer cell survival under conditions of metabolic stress (15). Its expression is associated with poor prognosis in non-small cell lung and cervical cancers (16–18). Although the mechanisms associated with its antiapoptotic activity have not been clearly elucidated, AAC-11 contains several protein interaction domains, including a leucine zipper (LZ) domain (19), and has been proposed to repress apoptotic effectors, such as E2F1 (20), Acinus (21), and caspase 2 (22). Synthetic peptides based on the LZ domain sequence of AAC-11 were previously shown to be cytotoxic to cancer cells both *in vitro* and in an *in vivo* mouse model of melanoma (23, 24) or acute leukemia (25).

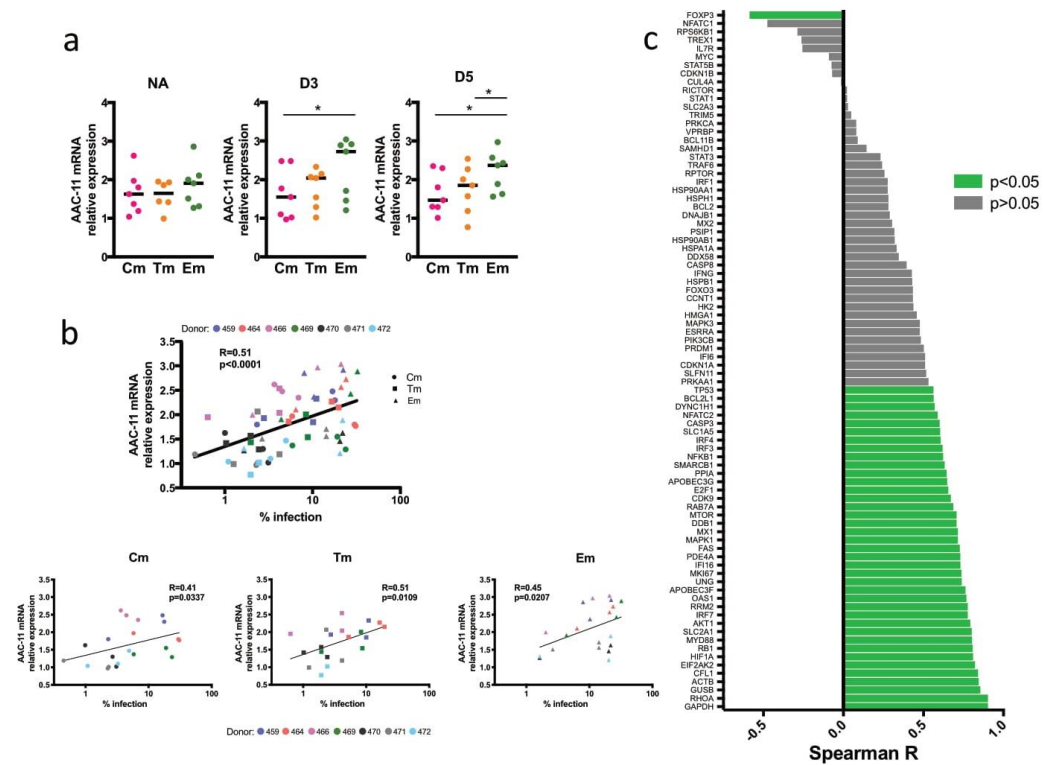
We explore here whether AAC-11-derived peptides could, similarly to its action against cancer cells, induce the elimination of HIV-1-infected cells. We found that AAC-11-derived peptides were preferentially cytotoxic for CD4<sup>+</sup> T cells targeted by HIV-1. In contrast, cells escaping the cytotoxic action of the peptides were resistant to HIV-1 replication. These results offer proof of principle that some characteristics of the cells targeted by the virus could be antagonized to counteract infection.

## RESULTS

**AAC-11-derived peptides display antiviral activity against diverse HIV-1 strains and simian immunodeficiency virus (SIV).** We previously analyzed the susceptibility of memory CD4<sup>+</sup> T cell subpopulations (Cm, Tm, and Em cells) to HIV-1 infection (7). We also analyzed the expression, at the time of infection, of a panel of 96 genes related to T cell differentiation, function, and survival, as well as restriction and HIV-facilitating factors (<https://data.mendeley.com/datasets/vfj3r27gnf/1>). Among other genes, we found in *post hoc* analysis that the expression of antiapoptotic factor AAC-11 increased progressively with the stage of differentiation of memory CD4<sup>+</sup> T cells (Cm < Tm < Em) and was correlated with infection in these subsets (Fig. 1a and b). The expression of AAC-11 was strongly correlated with the expression of multiple genes related to cell metabolism (e.g., SLC1A5, MTOR, HIF1A, GUSB, and GAPDH [glyceraldehyde-3-phosphate dehydrogenase] genes) and the cell cycle (e.g., RHOA, RB1, MAPK1, and MKI67 genes) and genes known to be necessary for different steps of the HIV replication cycle (RRM2, SAMRBC1, DDB1, CFL1, ACTB, CDK9, and NFKB1 genes) (26–42) (Fig. 1c). These results suggest that the AAC-11 antiapoptotic pathway is upregulated in cells that offer an optimal environment for HIV-1 replication.

We sought to antagonize the action of AAC-11 by using a panel of synthetic peptides derived from the LZ domain located in its alpha helix 18 (19) fused to a cell-penetrating sequence of the antennapedia protein of *Drosophila melanogaster* (also commonly known as penetratin and referred to as RK16 here) (Fig. 2a), which can be

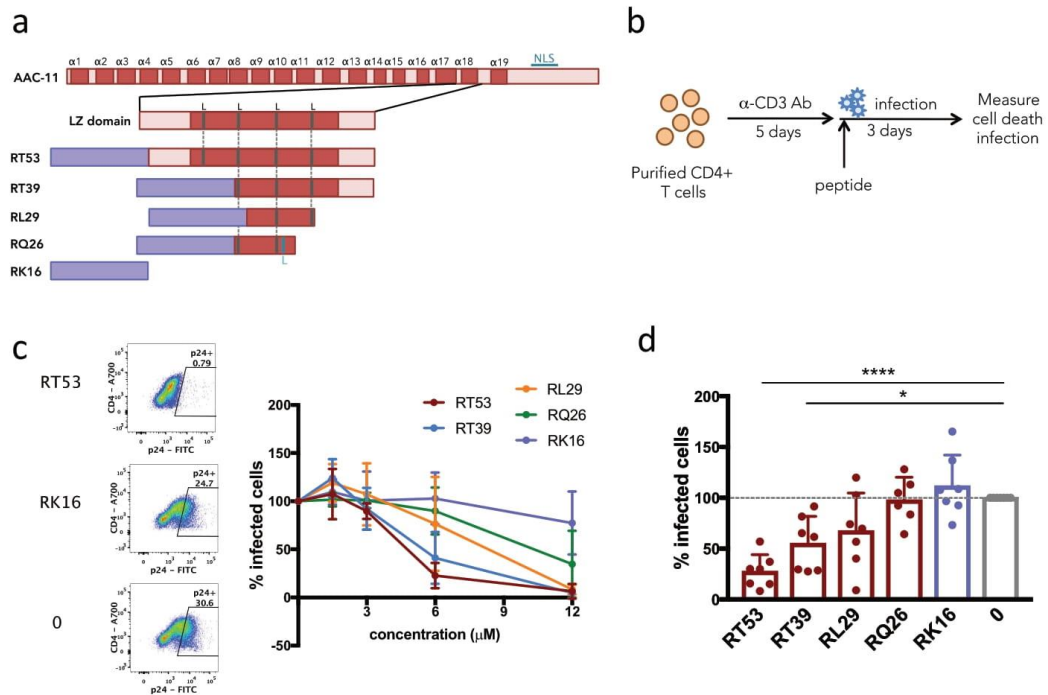




**FIG 1** AAC-11 gene expression correlates with the proportion of infection in each memory CD4<sup>+</sup> T cell subset and is associated with expression of various cell cycle and metabolism genes. (a) Nonactivated (NA) or activated (day 3 [D3] and 5 [D5]) CD4<sup>+</sup> T cell memory subsets (Cm, Tm, and Em) were analyzed for the expression of AAC-11 at the time of infection. Medians are indicated by horizontal lines. (b) Nonactivated or activated (day 3 and 5) Cm, Tm, and Em subsets were analyzed for levels of AAC-11 gene expression and correlated with the percentage of infected cells in each subset at day 3 postinfection. (c) Correlation of AAC-11 gene expression with other genes in a 96-gene array. Bars indicate positive and negative Spearman correlation coefficients. \*,  $P \leq 0.05$ .

used as an intracellular delivery method for its cargo (43). These AAC-11-derived peptides act as competitive inhibitors of protein-protein interactions, can prevent AAC-11 antiapoptotic activities, and have shown antitumor activities *in vitro* and *in vivo* (23–25). RT53 spanned the entire length of the AAC-11 LZ domain (Fig. 2a), and the other peptides spanned progressively smaller regions of the LZ domain. We applied these peptides to activated primary human CD4<sup>+</sup> T cells (44) immediately after infection with HIV-1 and measured infection levels 3 days later (Fig. 2b). AAC-11-derived peptides reduced HIV-1 infection in a concentration-dependent manner (Fig. 2c). Shorter peptides were less effective at inhibiting infection (Fig. 2c and d). RK16, the penetratin, alone did not show any antiviral effect even at the highest concentration tested. We observed a rapid drop in the proportion of infected cells at a 6  $\mu\text{M}$  peptide concentration for RT53 and RT39, the peptides exhibiting the strongest antiviral effects. At this concentration, RT53 was most potent at suppressing infection (50% effective concentration [ $EC_{50}$ ] = 4.19  $\mu\text{M}$ ) (Fig. 2d), indicating that the entire length of the LZ domain of AAC-11 was necessary for full peptide potency. We, therefore, proceeded to work with RT53 at a 6  $\mu\text{M}$  concentration.

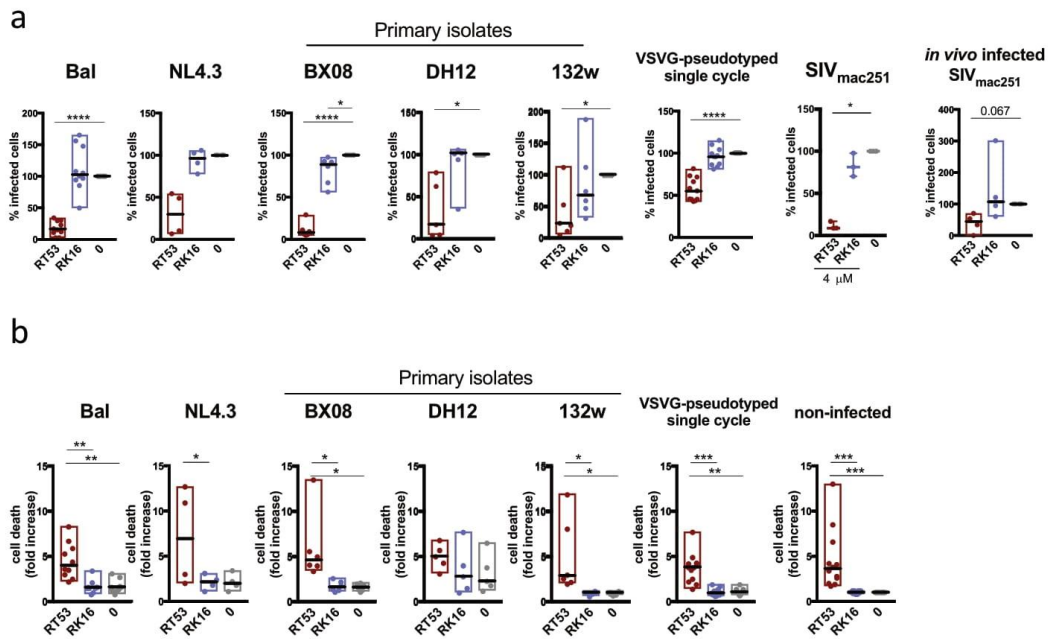
We next tested if RT53 was able to inhibit infection of distinct HIV-1 strains (Fig. 3a). Both strains R5 (Bal) and X4 (NL4.3), as well as primary isolates (BX08, DH12, and 132w)



**FIG 2** AAC-11-derived peptides inhibit HIV-1 infection. (a) Schematic representation of AAC-11-derived peptides showing the LZ domain. NLS, nuclear localization signal. (b) Experimental scheme of treatment of infected cells with AAC-11-derived peptides. (c) (Left) Representative flow cytometry plot of p24 staining in live untreated cells (0) or cells treated with 6  $\mu$ M RT53 or RK16. (Right) Dose-response curve of the effect of AAC-11-derived peptides on HIV-1 infection in living cells. (d) Proportion of infection compared to nontreated control among living cells infected with HIV-1 Bal and treated with 6  $\mu$ M the indicated peptides. \*,  $P \leq 0.05$ ; \*\*\*\*,  $P \leq 0.0001$ .

and single-cycle vesicular stomatitis virus glycoprotein (VSVG)-pseudotyped HIV-1 particles, were inhibited, albeit less efficiently for the last. RT53 was also able to inhibit *in vitro* infection of human CD4<sup>+</sup> T cells with SIV<sub>mac251</sub>. Moreover, it inhibited viral spread from splenic CD4<sup>+</sup> T cells of cynomolgus macaques previously infected with SIV<sub>mac251</sub> (Fig. 3a). The inhibition of infection in both human and macaque cells is coherent with the high conservation of AAC-11 across species (20) and 100% homology between humans and macaques. Again, RK16 alone had no inhibitory effect on any of the viruses tested. Overall, our results showed that AAC-11-derived peptides were able to impair HIV-1 and SIV infection of CD4<sup>+</sup> T cells *in vitro*, as well as spread from *in vivo*-infected CD4<sup>+</sup> T cells.

**Blockage of HIV-1 infection by AAC-11-derived peptides is associated with cell death.** Since the AAC-11 peptides have been shown to induce cytotoxicity in cancer cells (21), we next evaluated the amount of cell death in our cultures. Indeed, we saw that all the peptides caused various rates of cell death (Fig. 4a). Moreover, we found that the proportions of cell death caused by different peptides in different donors were negatively correlated with the proportions of HIV-infected cells in the cultures (Fig. 4b). As before, RT53 was most potent at causing cell death. Interestingly, RT53 induced similar death rates in cells infected with various HIV-1 strains and in cells that had not been challenged with HIV-1 (Fig. 3b and 4a). This indicated that some CD4<sup>+</sup> T cells had preexisting sensitivity to the action of the peptides that was not dependent on infection.

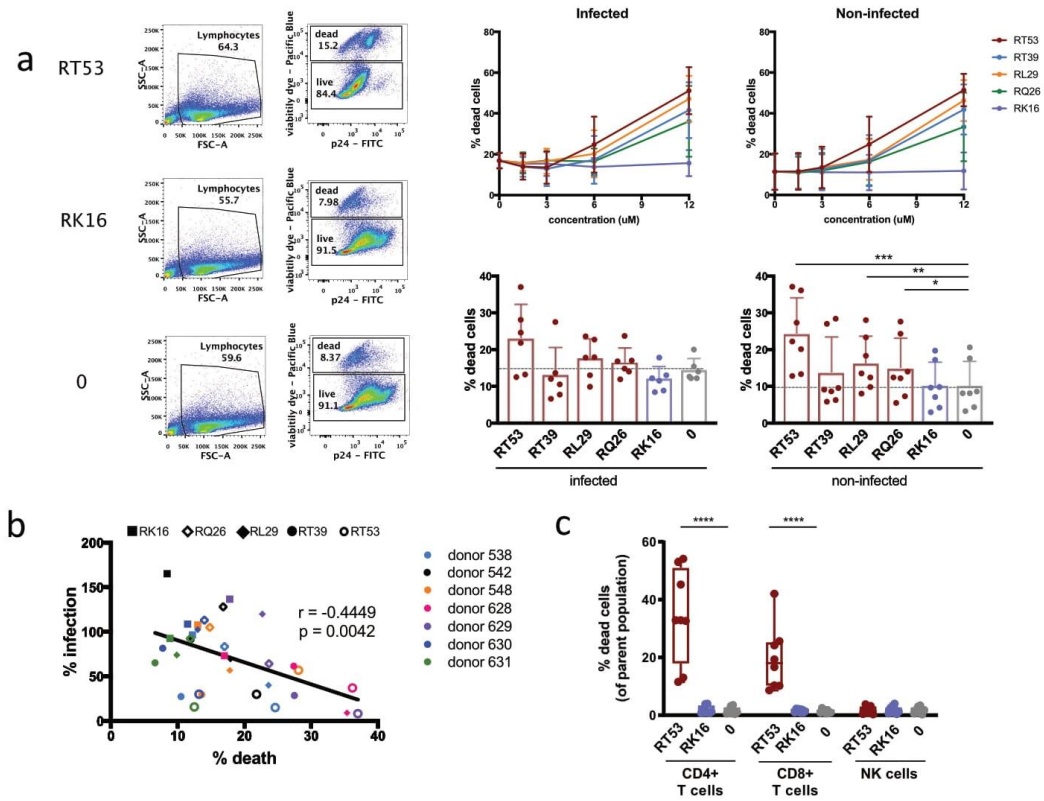


**FIG 3** RT53 inhibits infection of diverse HIV-1 strains and SIV. Activated primary CD4<sup>+</sup> T cells were infected with different HIV-1 strains and incubated with 6  $\mu$ M RT53 or RK16 for 3 days. (a) Infection was measured by flow cytometry (intracellular p24) or ELISA (p24/p27) in supernatants. (b) Cell death was measured by flow cytometry (LIVE/DEAD violet viability dye). Values are expressed as a range (minimum-maximum) with medians indicated. Each symbol represents experiments with cells from a different donor. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ ; \*\*\*\*,  $P \leq 0.0001$ .

To explore the specificity of action of AAC-11-derived peptides, we analyzed if RT53 induced cell death in other cell types. We found that CD8<sup>+</sup> T cells were susceptible to the action of RT53, although to a lesser extent than CD4<sup>+</sup> T cells ( $P = 0.039$ ), while NK cells were resistant to the cytotoxicity of RT53 ( $P = 0.0078$ ) (Fig. 4c). Therefore, RT53 did not have a general cytotoxic effect on all lymphocytes. On the other hand, RT53 induced cytotoxicity in highly susceptible Jurkat and SupT1 human CD4<sup>+</sup> T cell lines, but it did not affect the proportion of infected cells (data not shown), not recapitulating the selective effect observed in primary CD4<sup>+</sup> T cells, further suggesting that the link between peptide-induced cytotoxicity and antiviral activity is due to particular properties of primary CD4<sup>+</sup> T cells that favor HIV replication.

Together, these results show that AAC-11 did not exert a nonspecific cytotoxic action but affected particular cell subsets. Primary CD4<sup>+</sup> T cells preferentially targeted by HIV-1 for infection had enhanced sensitivity to the actions of AAC-11-derived peptides. Hence, AAC-11-derived peptides may induce selective elimination of HIV-1 target cells.

**Cell death induced by AAC-11-derived peptides is associated with K<sup>+</sup> efflux and mitochondrial depolarization.** We next investigated the mechanism of the RT53-mediated effect. We started by evaluating the kinetics of RT53 action. We used real-time flow cytometry to read out changes in several molecular indicators associated with cell death as a function of time (45). We found that treatment of cells with RT53 induced a sharp and rapid increase in the surface levels of phosphatidylserine (PS) (as determined by binding to annexin V-fluorescein isothiocyanate [FITC]), classically associated with apoptosis induction (Fig. 5a). Additionally, in a subset of RT53-treated CD4<sup>+</sup> T cells, we also observed decreases in the intracellular levels of K<sup>+</sup> ions (revealed

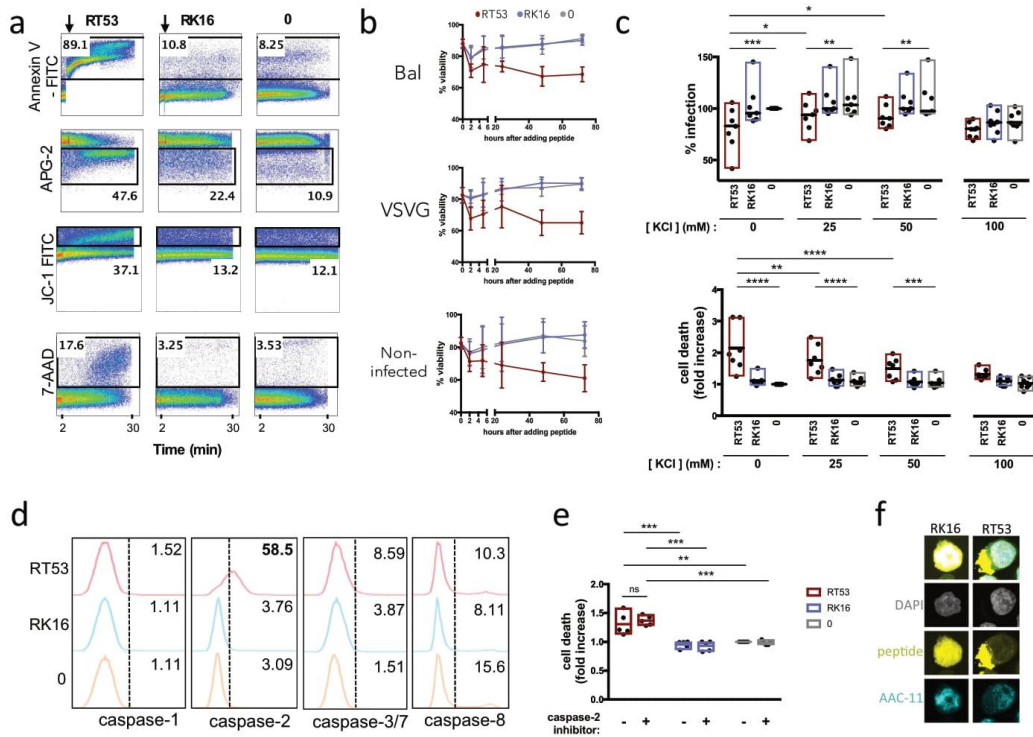


**FIG 4** Decrease in HIV-1 infection is associated with cell death. (a) Example of flow cytometry gating strategy to evaluate cell death in cultures. *In vitro*-activated primary CD4<sup>+</sup> T cells were incubated with various concentrations of AAC-11-derived peptides after infection with HIV-1 Bal, and cell death was evaluated by flow cytometry on day 3 of infection. (b) Proportions of infected cells among live cells versus cell death in different donors' CD4<sup>+</sup> T cells incubated with 6  $\mu$ M the indicated peptides. (c) PBMCs were treated with 6  $\mu$ M RT53 or RK16 for 5 h, washed, stained for markers of CD4<sup>+</sup> CD8<sup>+</sup> T cells and NK cells and viability dye, and assessed by flow cytometry. The bars represent the minimum-maximum range, with medians indicated. Each symbol represents experiments with cells from a different donor. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ ; \*\*\*\*,  $P \leq 0.0001$ .

by the fluorescent K<sup>+</sup> indicator asanate potassium green 2 [APG-2]), the efflux of which has been linked to the activation of the cell death machinery (46–48), and in mitochondrial depolarization, evaluated by the increase in green fluorescence of the mitochondrial membrane potential dye JC-1 (Fig. 5a). These events were accompanied by an increase in DNA labeling with 7-aminoactinomycin D (7-AAD), a classical marker for dead cells. Cell death peaked at 30 min after treatment with the RT53 peptide in both noninfected and infected cells, with no significant increase at later time points (Fig. 5b).

To investigate if cell death caused by the peptides was indeed responsible for the observed antiviral effect, we sought to revert RT53-induced cytotoxicity by antagonizing intracellular K<sup>+</sup> efflux with increasing KCl concentrations in the culture medium (49). Noninfected CD4<sup>+</sup> T cells or cells that had been preinfected with VSVG-pseudotyped HIV particles were incubated with RT53 or RK16 for 5 h in the presence of increasing extracellular K<sup>+</sup> concentrations of up to 100 mM KCl supplementing the cell culture medium (Fig. 5c). We observed a KCl concentration-dependent decrease in RT53-mediated cell death relative to the RK16-treated or untreated condition, which

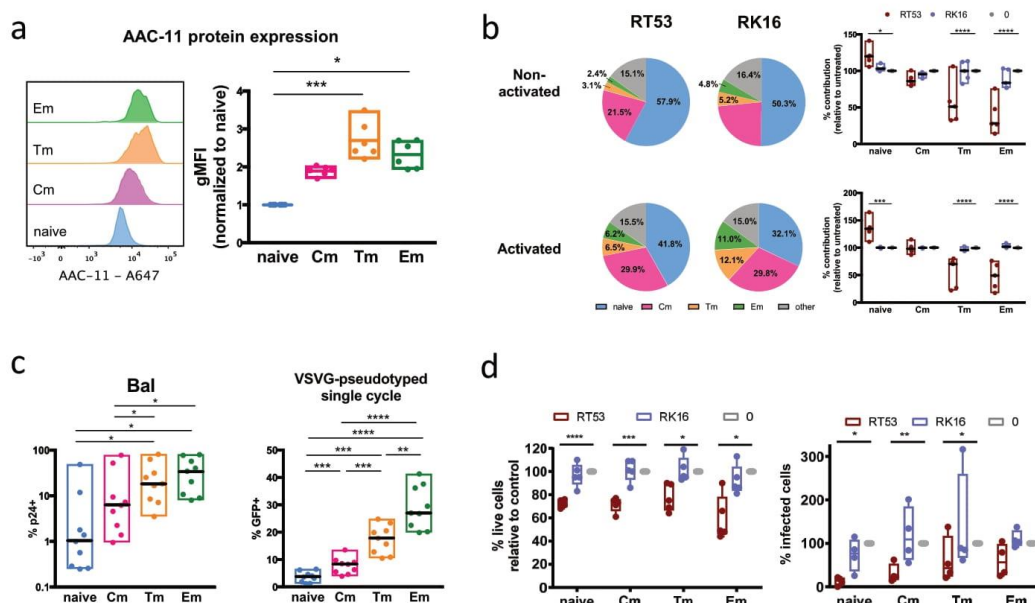




**FIG 5** RT53-mediated cell death is rapid and is associated with K<sup>+</sup> efflux and mitochondrial depolarization. (a) Cells were placed in annexin V-FITC and 7-AAD staining buffer or loaded with a fluorescent K<sup>+</sup> indicator (APG-2) or a mitochondrial membrane potential probe (JC-1) and run on a flow cytometer to acquire fluorescence over time. The time point of peptide addition (after 2 min of acquisition) is indicated with an arrow. (b) Infected cells were incubated with 6  $\mu$ M RT53 or RK16, and viability was determined with trypan blue at the indicated time points. (c) Cells preinfected with VSVG-pseudotyped HIV-1 particles (day 3 of infection) were incubated with 6  $\mu$ M RT53 or RK16 in the presence of various concentrations of KCl for 5 h. Infection and cell death were then evaluated by flow cytometry. The symbols represent experiments with cells from different donors. The bars represent the minimum-maximum range, with medians indicated. (d) Cells were incubated with 6  $\mu$ M RT53 or RK16 for 2 h and stained with probes for active caspases. (e) Cells were incubated with caspase 2 FAM-VDVAD-FMK probe and pulsed with 6  $\mu$ M RT53 or RK16. Cell death was measured 5 h later by flow cytometry. (f) Immunofluorescent detection of RT53/RK16-Rd and AAC-11 in CD4<sup>+</sup> T cells. Cells were incubated with 6  $\mu$ M RT53/RK16-Rd for 2 h, washed, immobilized on polylysine-coated coverslips, fixed, and stained with antibodies for AAC-11 and DAPI. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ ; \*\*\*\*,  $P \leq 0.0001$ .

was accompanied by a progressive increase in the proportion of infected CD4<sup>+</sup> T cells recovered at the end of culture. No differences were observed in the rates of cell death and the percentages of infected cells between RT53 and RK16 or a control when the cultures were done in the presence of 100 mM KCl, although we note that at this concentration, the rates of infection were overall lower than in CD4<sup>+</sup> T cells cultured in the absence of extra KCl (Fig. 5c).

We then evaluated if RT53 treatment had an impact on the activation of caspases, known activators and effectors of cell death. Of note, caspase 2 has been recently reported to be repressed by AAC-11 (22). Upon treatment with the AAC-11-derived peptide, we did not observe significant activation of caspase 1, caspase 3/7, and caspase 8, but we saw strong activation of caspase 2 (Fig. 5d). Nevertheless, inhibition of caspase 2 activation did not lead to a decrease in cell death (Fig. 5e), suggesting that activation of caspase 2 was not the main mechanism responsible for cell death in our system. Similar results were obtained with inhibition of caspase 1, caspase 3/7, and caspase 8 (data not shown). These results are in agreement with a caspase-independent mechanism of cytotoxicity induced by RT53 in cancer cells (24).



**FIG 6** RT53 preferentially targets Tm and Em cells to cell death. (a) *In vitro*-activated CD4<sup>+</sup> T cells were stained for markers of CD4<sup>+</sup> T cell memory subsets and AAC-11. (b) *In vitro*-activated or nonactivated CD4<sup>+</sup> T cells were pulsed with 6  $\mu$ M RT53 or RK16 for 5 h, harvested, and stained for markers of CD4<sup>+</sup> T cell memory subsets. (c) Levels of HIV-1 Bal or VSVG-pseudotyped single-cycle HIV particle infection were quantified by intracellular p24 staining in different CD4<sup>+</sup> T cell subsets by flow cytometry. (d) *In vitro*-activated CD4<sup>+</sup> T cells were sorted by FACS into naive, Cm, Tm, and Em subsets. Each subset was pulsed with 6  $\mu$ M RT53 or RK16 for 24 h, and viability was measured by flow cytometry with LIVE/DEAD violet viability dye. In parallel, sorted cells were infected with HIV-1 Bal in the presence or absence of HIV-1 Bal, and rates of infection were determined on day 3 postinfection by intracellular p24. Viability and infection levels are expressed in relation to the untreated condition for each individual. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ ; \*\*\*\*,  $P \leq 0.0001$ .

RT53 was previously shown to mediate its cytotoxic effect through a membranolytic action by means of interaction with an unknown AAC-11 partner (23, 24). We therefore determined the cellular localization of RT53 and RK16 conjugated to rhodamine (Rd). As expected, RK16-Rd was found in the cytoplasm of the CD4<sup>+</sup> T cells (Fig. 5f), while RT53-Rd localized predominantly to the cell membrane. These studies were carried out at a 6  $\mu$ M peptide concentration, but similar results were found at subcytotoxic peptide concentrations of 2 and 4  $\mu$ M (data not shown). Therefore, our results indicate that, similar to cancer cells, RT53 could be causing cell death in primary CD4<sup>+</sup> T cells by membranolytic action.

Overall, our results show that RT53 induced rapid cell death in a subset of CD4<sup>+</sup> T cells, possibly through a membranolytic mechanism. The impairment of peptide-induced cell death abrogated the antiviral activity of the peptides, which provides a direct link between the peptide cytotoxic effect and its capacity to inhibit HIV infection.

**RT53 preferentially depletes effector and transitional memory CD4<sup>+</sup> T cell subpopulations.** Our previous results showed that AAC-11 mRNA expression was upregulated with CD4<sup>+</sup> T cell differentiation (Fig. 1). We have now found that the expression of AAC-11 at the protein level also increased with T cell differentiation (Fig. 6a). We therefore wondered if RT53 might have distinct impacts on different CD4<sup>+</sup> T cell subsets. RT53 treatment changed the relative contribution of CD4<sup>+</sup> T cell subsets to the total pool of CD4<sup>+</sup> T cells compared to nontreated or RK16-treated conditions (Fig. 6b). Naive CD4<sup>+</sup> T cells were enriched upon treatment with RT53, while the more differentiated Tm and Em CD4<sup>+</sup> T cells were depleted. Although activation *in vitro* changed the relative contribution of CD4<sup>+</sup> T cell subsets to the pool of CD4<sup>+</sup> T cells

somewhat, RT53 had similar impacts on CD4<sup>+</sup> T cell subsets when we used nonactivated or *in vitro*-activated cells (Fig. 6b). As previously reported (7), under our experimental conditions, naive CD4<sup>+</sup> T cells were highly resistant to infection with both VSVG-pseudotyped HIV-1 particles and HIV-1 Bal (Fig. 6c). Susceptibility to infection increased with T cell differentiation, with Em and Tm cells showing the highest infection levels (Fig. 6c). Thus, our results showed that RT53 preferentially targeted differentiated memory cells, which had heightened susceptibility to HIV infection.

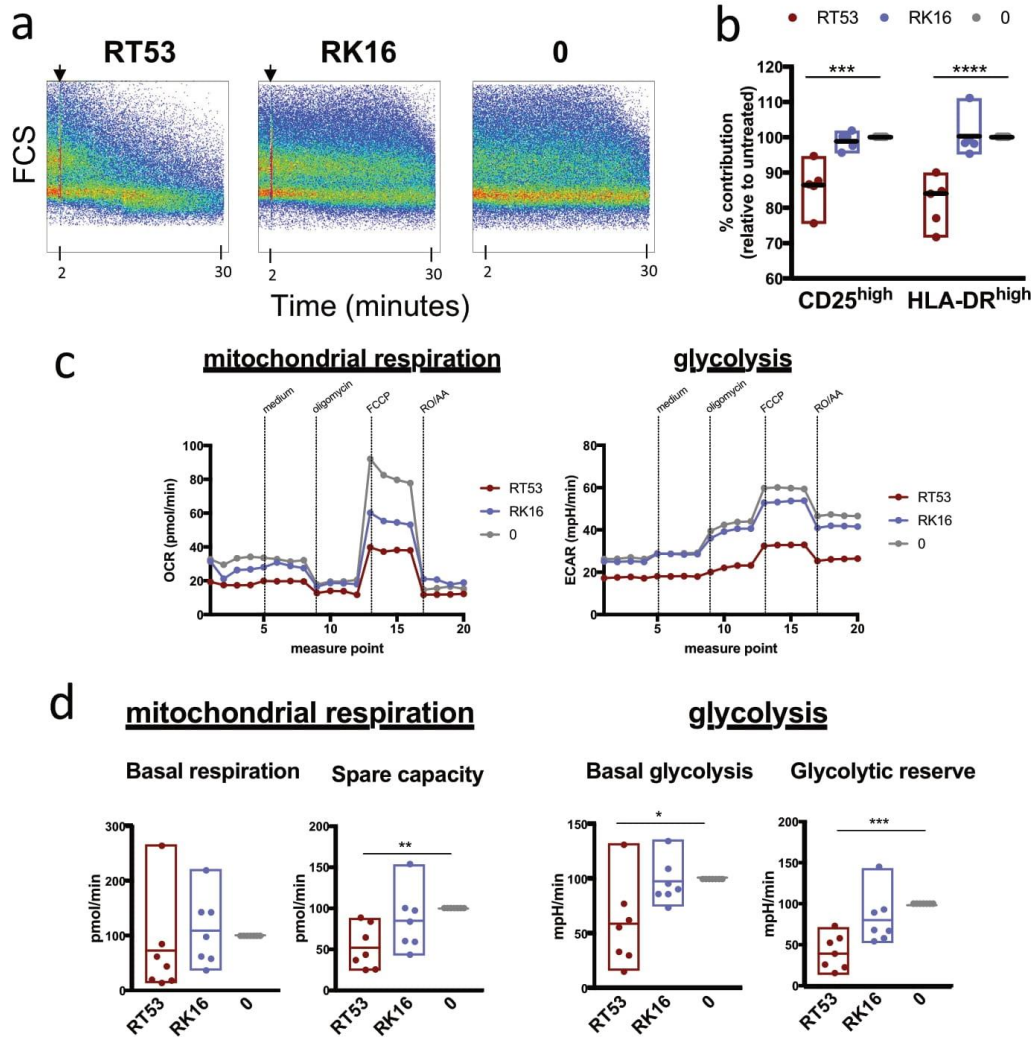
However, not all Em and Tm cells were depleted after RT53 treatment, and some extent of peptide-induced cell death could also be observed in other CD4<sup>+</sup> T cell subsets, including naive cells. We therefore separately tested the impacts of RT53 treatment on isolated naive, Cm, Tm, and Em CD4<sup>+</sup> T cells. We confirmed that RT53 induced cell death in each of the CD4<sup>+</sup> T cell subsets (Fig. 6d). Moreover, RT53 treatment decreased HIV-1 infection in all CD4<sup>+</sup> T cell subsets. Overall, these results show that RT53 targeted cells with specific characteristics that were strongly enriched, but not exclusively found, within more differentiated subsets. Of note, the cells targeted by RT53 within each subset were susceptible to HIV-1 infection.

**RT53 targets metabolically active cells.** We next aimed to identify the characteristics of the CD4<sup>+</sup> T cells that were sensitive to the cytotoxic action of RT53. RT53 was previously shown to induce death of cancer cells during metabolic stress (50). Cancer cells rely on a high level of metabolic activity for adequate supply of biosynthetic intermediates for their life cycle, and similar conditions favor HIV-1 replication in CD4<sup>+</sup> T cells (7). We therefore analyzed if the differential sensitivities of CD4<sup>+</sup> T cells to RT53 treatment were related to differences in cellular metabolism. Following treatment of CD4<sup>+</sup> T cells with RT53, but not RK16, we observed the disappearance of larger CD4<sup>+</sup> T cells (determined by forward scatter [FSC] in flow cytometry) (Fig. 7a) and a decrease in the proportions of CD25<sup>high</sup> and HLA-DR<sup>high</sup> cells (Fig. 7b). We next analyzed the metabolic activity (glycolysis, measured by the extracellular acidification rate [ECAR], and oxidative phosphorylation [OXPHOS], measured by the oxygen consumption rate [OCR]) of living CD4<sup>+</sup> T cells in nontreated cells or cells treated with RT53/RK16 peptide (Fig. 7c). We found that the cells that survived RT53 treatment had lower mitochondrial respiration (spare respiratory capacity) and glycolysis (basal ECAR and glycolytic reserve [ $\Delta$ ECAR after addition of the uncoupler of mitochondrial oxidative carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP)]) than cells that had not been treated or were treated with RK16 (Fig. 7d). We therefore concluded that CD4<sup>+</sup> T cells with the highest metabolic activity were more sensitive to the action of AAC-11-derived peptides.

**RT53 depletes the pool of cells permissive to HIV-1 replication.** To further confirm that RT53 was killing HIV-1-susceptible target cells and selecting for cells resistant to viral replication, we treated activated CD4<sup>+</sup> T cells with RT53 or RK16 for 24 h, sorted live cells from dead cells, and infected the sorted live cells (Fig. 8a). We found that the cells that survived RT53 treatment were less susceptible to both HIV-1 Bal and VSVG-pseudotyped HIV-1 particles (Fig. 8a), although the inhibition was more marked for the fully competent virus. Importantly, we did not see any further increase in cell death compared to control conditions in sorted living cells.

We next sought to determine which stage of the viral replication cycle was blocked in cells surviving RT53 treatment. RT53 induced a decrease in the proportion of CCR5<sup>high</sup> cells (Fig. 8b), although this difference did not appear sufficient to explain the level of HIV inhibition found. Moreover, RT53 did not change CCR5 cell surface distribution in the cells that survived treatment with RT53 (Fig. 8b). RT53 was still able to block infection when added 24 h after the culture of CD4<sup>+</sup> T cells with HIV-1 Bal (Fig. 8d). We next measured the levels of total (as a measure of reverse transcription) and integrated viral DNA at various time points postinfection with VSVG-pseudotyped HIV-1 particles in RT53/RK16-treated or untreated cells (Fig. 8e). The levels of both total and integrated HIV-1 DNA drastically increased as a function of time in RK16-treated and untreated cells, but not in RT53-treated cells. These results indicate that the virus is unable to complete early steps of its life cycle in cells surviving RT53 treatment and that these cells are nonpermissive to viral





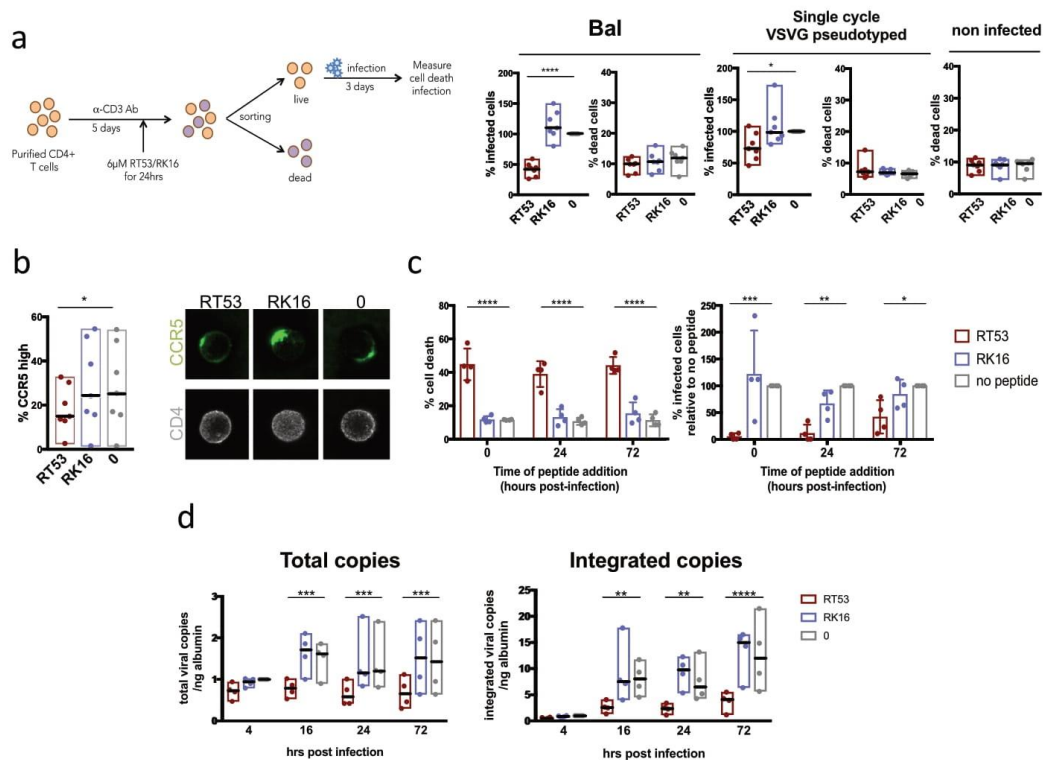
**FIG 7** RT53 preferentially targets activated and most metabolically active cells. (a) Example of RT53 treatment preferentially targeting large cells (evaluated by the FCS parameter by real-time flow cytometry). (b) *In vitro*-activated cells were pulsed with 6  $\mu$ M RT53 or RK16 for 5 h. The relative surface expression of CD25 and HLA-DR by flow cytometry compared to the no-peptide condition is shown. (c) OCR and ECAR values of CD4<sup>+</sup> T cells from one representative donor. (d) Relative basal mitochondrial respiration (basal respiration), spare mitochondrial capacity (spare capacity), basal glycolysis (basal ECAR), and glycolytic reserve (ECAR after FCCP) obtained with cells from 7 donors compare to the no-peptide condition (0). Values are expressed as a minimum-maximum range, with medians indicated. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ ; \*\*\*\*,  $P \leq 0.0001$ .

replication. Thus, RT53 treatment killed cells in which the virus was able to establish productive infection while allowing the survival of HIV-1-resistant cells.

## DISCUSSION

In this study, we showed that peptides derived from the LZ region of the antiapoptotic factor AAC-11 inhibited HIV-1 infection of primary CD4<sup>+</sup> T cells. In particular, we





**FIG 8** RT53-surviving cells are resistant to HIV infection. (a) *In vitro*-activated CD4<sup>+</sup> T cells were incubated with 6 μM RT53 or RK16 for 24 h. Live cells were then sorted from dead cells using FACS, and the live cells were infected with the indicated HIV-1 strains. Cell death and infection were evaluated by flow cytometry 3 days later. (b) Proportion of CCR5<sup>high</sup>-expressing cells and CCR5 cell surface distribution among *in vitro*-activated CD4<sup>+</sup> T cells after 5 h of treatment with 6 μM RT53 or RK16. (c) Antiviral effect of RT53 when added at different times after infection. Activated CD4<sup>+</sup> T cells were infected with HIV-1 Bal, and 6 μM RT53 or RK16 was added at the indicated times postinfection. The levels of cell death and infection were quantified by flow cytometry on day 3 postinfection. (d) Total and integrated viral copies evaluated at various time points postinfection in cells infected with VSVG-pseudotyped HIV-1 particles and treated with 6 μM RT53 or RK16. The values are normalized to the untreated condition 4 h postinfection and expressed as minimum-maximum ranges with medians indicated. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ ; \*\*\*,  $P \leq 0.001$ ; \*\*\*\*,  $P \leq 0.0001$ .

found that RT53 was able to block infection with a large panel of laboratory-adapted and primary viral strains by inducing the death of CD4<sup>+</sup> T cells that offered the best conditions to sustain HIV-1 replication.

Although sensitivity to RT53 was more pronounced in highly differentiated CD4<sup>+</sup> T cell subsets, RT53 treatment eliminated a fraction of the cells within each cell subset, rendering the remaining cells resistant to HIV-1 replication. RT53 depleted highly metabolic cells, which is in agreement with the observation that a rich metabolic environment is necessary for HIV replication (7). Upon antigenic stimulation, CD4<sup>+</sup> T cells upregulate their metabolic activities, in particular glucose metabolism, to cope with the bioenergetic demands necessary to perform their functions. Thus, naive and central memory CD4<sup>+</sup> T cells are typically smaller and have decreased metabolic activities compared to more differentiated and activated subsets. However, we have recently shown that some cells with enhanced metabolic activity can be found even among phenotypically quiescent naive T cells and that these cells are susceptible to HIV-1 infection (7). Here, we found that there was a close association between AAC-11 peptide-induced cell death and inhibition of HIV infection. Not only did the rates of cell

death induced by AAC-11 peptides correlate with the extent of HIV inhibition, but precluding cell death by increasing extracellular levels of  $K^+$  also restored the infectibility of  $CD4^+$  T cells. Cellular metabolism and cell death are deeply entangled. Metabolic arrangements are necessary to sustain long-term memory cells. External signals provided by T cell receptor activation or growth factors, such as interleukin 2 (IL-2) and IL-7, promote an antiapoptotic state of the cell by increasing the levels of metabolite transporters (e.g., Glut1) that ensure the supply of nutrients necessary to sustain the bioenergetic demands of the cell (51, 52). The absence of these signals provokes a limitation in the influx of nutrients that results in metabolic stress and the activation of cell death pathways. Our results indicate that AAC-11-derived peptides may tilt the equilibrium of highly metabolic cells toward cell death, even among less differentiated cells with a stronger antiapoptotic basal state.

Although the antiapoptotic action of AAC-11 is well documented, the molecular mechanisms underlying its activity are still unclear. AAC-11 possesses several domains predicted to be responsible for protein-protein interactions (19), and its expression may interfere with different mechanisms of cell death. The peptides that we tested here were designed to mimic the heptad leucine repeat region of AAC-11 and to be used as competitive inhibitors that abrogate the interaction of AAC-11 with its partners. In tumor cells, physical interaction between the LZ domain of AAC-11 and Acinus prevents Acinus proapoptotic cleavage by caspase 3. In our study, we did not observe significant changes in the expression of Acinus upon treatment with AAC-11-derived peptides (data not shown), and caspase 3 activity was not significantly detected under our experimental conditions, suggesting that this pathway was not the major contributor to the T cell death observed in our experiments. In contrast, we found that treatment with RT53 induced a strong increase in caspase 2 activity. Although AAC-11 has been shown to physically bind to the caspase recruitment domain of caspase 2, preventing its autocleavage (22), the activation of this caspase seen in our experiments was probably due to a side effect of the decrease in intracellular  $K^+$  levels caused by the peptides (22, 53). The inhibition of caspase 2 activity also did not lead to the restoration of cell viability, suggesting that the mechanism of cell death induced by RT53 is independent of caspase 2 activation. RT53 was previously seen to localize to the plasma membrane compartment of cancer cells and was reported to have a membranolytic mechanism of action. We have also observed the peptide's localization to this cellular compartment in primary  $CD4^+$  T cells. The fast kinetics of the peptide's activity also point to action through rearrangement of preexisting cellular factors probably found at the cell's plasma membrane and enriched in metabolically active cells.

Previous analyses have ruled out the nonspecific detergent-like cell death mechanism of RT53 (24). Indeed, we also found here that RT53 was cytotoxic to only some subsets of peripheral blood mononuclear cells (PBMCs), thus confirming the specificity of RT53 action, possibly through a membrane partner absent from certain cells. Interestingly, we also observed that RT53 did not have an antiviral effect despite observable cytotoxicity in the  $CD4^+$  Jurkat and SupT1 T cell lines. Immortalized cell lines are known to have perturbed survival and metabolic pathways, thus providing a more favorable molecular environment for HIV-1 replication. This observation also points to the existence of a specific survival pathway used by HIV-1 in primary  $CD4^+$  T cells perturbed by RT53.

Of note, although we observed consistent inhibition of infection with VSVG-pseudotyped HIV-1 single-cycle particles upon treatment with AAC-11 peptides, the inhibition was more important when we used wild-type (WT) viruses, either R5 or X4. There are several possible explanations for this. First, the additional block to HIV infection observed when we used WT viruses could be due to a cumulative effect of multiple infection cycles. Second, cells selected upon treatment with AAC-11 may be more resistant to WT virus. For instance, some cells expressing high levels of CCR5 were depleted upon treatment with RT53. We also cannot exclude the possibility that treatment with AAC-11-derived peptides induced further viral restriction in the cells that survived. Finally, infection with WT HIV might induce cellular changes that could

increase the susceptibility of infected cells to the action of AAC-11-derived peptides. For instance, HIV has been shown to induce metabolic changes in infected cells and, in particular, an increase in the expression of Glut1 to -3 (54).

Our results showed that AAC-11-derived peptides were active against CD4<sup>+</sup> T cells that were particularly susceptible to HIV-1 infection. It remains to be established if this sensitivity to the peptides remains in persistently infected cells. However, our results suggest that sensitivity to the action of RT53 decreased somewhat 72 h after infection, suggesting HIV-1-mediated modification of the cell death pathways in infected cells, as has been shown for macrophages (55). The mechanisms by which an infected cell persists are not completely clear, as HIV has cytopathic effects that lead to the death of infected CD4<sup>+</sup> T cells. It has been recently reported that persistent latently infected CD4<sup>+</sup> T cells in which the virus has been reactivated are intrinsically resistant to killing by HIV-specific CD8<sup>+</sup> T cells (56), which indicates that the persistent reservoir might be seeded in cells programmed to resist cell death. BIRC5 (also known as Api4 or survivin), a member of the inhibitor of apoptosis protein family, is upregulated in CD4<sup>+</sup> T cells during HIV-1 infection and contributes to the persistence of infected cells (57). Upregulation of BIRC5 on infected CD4<sup>+</sup> T cells was triggered by OX40, a costimulatory receptor that promotes T cell differentiation and survival (58, 59) and contributes to the metabolic boost necessary for T cell activation (60). It is thus possible that HIV-1 exploits/activates several cellular survival programs associated with T cell activation and metabolic activity to ensure its persistence.

Although further studies will be necessary to directly evaluate this, our results suggest that HIV-1 may rely on the AAC-11-dependent antiapoptotic pathway for the establishment of productive infection in CD4<sup>+</sup> T cells. AAC-11 gene expression, besides strongly correlating with multiple genes involved in the regulation of cell metabolism, was also correlated with the expression of RRM2 (Fig. 1c), an enzyme that is critical for the *de novo* synthesis of deoxynucleoside triphosphates (dNTPs) and the depletion of which blocks HIV-1 infection in macrophages and dendritic cells (42, 61). AAC-11 mRNA levels also correlated with the expression of other genes that have been associated with the HIV replication cycle, in particular, trafficking (i.e., CFL1, DYNC1H1, and ACTB) and transcription (i.e., CDK9 and NFKB1). This suggests that AAC-11-expressing cells may offer an ideal environment for HIV-1 replication. AAC-11 prevents apoptosis of tumor cells under conditions of nutrient deprivation and in the absence of growth factors. We can therefore speculate that the AAC-11 survival pathway might play a similar role in promoting persistence of memory CD4<sup>+</sup> T cells and HIV infection. Like the mechanisms associated with HIV persistence, it is also unclear how some T cells survive to become long-lived memory cells. While most T cells undergo apoptosis at the end of the immune response when environmental signals wane, a few cells survive even in the absence of growth factors (62). We found that expression of the apoptosis-inhibitory protein AAC-11 increased with differentiation of memory CD4<sup>+</sup> T cells. Our results suggest that the AAC-11 survival pathway may be involved in the regulation of T cell immunity, a function that has not been previously described and deserves further detailed exploration.

Among current strategies under evaluation in pursuing a HIV cure, allogeneic hematopoietic stem cell transplantation has been shown to drastically reduce the viral reservoir (63). However, this is a risky intervention associated with severe immune ablation. We found that AAC-11 peptides blocked HIV infection through the preferential killing of "HIV-1-infectable" cells but preserved a significant fraction of T cell immunity, in particular, the less differentiated T cells. RT53 has been shown to be efficient *in vivo* in murine models of cancer with few adverse effects. Although its clinical application in the context of HIV infection remains uncertain, our results serve as a proof of concept that selective elimination of HIV-1 targets is a possible therapeutic strategy.

## MATERIALS AND METHODS

**Peptides, antibodies, and probes.** Peptides (Proteogenix) were received as dry powder and reconstituted with water for use. The peptide sequences are as follows: RT53, RQIKIWFQNRRMKWKKAKLNAEKLKDFKIRLQYFARGLQVYIRQLRLALQGKT; RT39, RQIKIWFQNRRMKWKKLQYFARGLQVYIRQLRLALQGKT; RL29, RQIKIWFQNRRMKWKKYFARGLQVYIRQL; RQ26, RQIKIWFQNRRMKWKKLQYFARGLQ; RK16, RQIKIWFQNRRMKWKK.

The antibodies and dyes used for flow cytometry and fluorescence-activated cell sorting (FACS) were as follows: LIVE/DEAD violet viability dye (ThermoFisher), CD3-phycoerythrin (PE) (clone SK7; Biolegend), CD4-A700 (clone OKT4; eBioscience), CD45RA-allophycocyanin (APC)-Cy7 (clone HI100; Biolegend), CCR7-PE-Cy7 (clone GO43H7; Biolegend), CD27-APC (clone M-T271; Miltenyi), CD25-PE-Dazzle 594 (clone M-A251; Biolegend), HLA-DR-peridinin chlorophyll protein (PerCP)-Cy5.5 (clone G46-6; Biolegend), CCR5-PE (clone 3A9; BD), and p24-FITC (clone KC57; Coulter). AAC-11 expression was analyzed by intracellular flow cytometry staining using a BD Cytofix/Cytoperm buffer set (BD) and anti-AAC-11 primary antibody (ab65836; Abcam) at 1/100 dilution, followed by a secondary antibody conjugated to A674 (ThermoFisher; A21244) at 1/1,000 dilution. Caspase activity was assayed with the following probes according to the manufacturer's instructions: caspase 1 660-YVAD-FMK probe (FLICA 660 caspase 1 assay kit; ImmunoChemistry Technologies LLC), caspase 2 6-carboxyfluorescein (FAM)-VDVAD-FMK probe (FAM-FLICA caspase 2 assay kit; ImmunoChemistry Technologies LLC), caspase 3/7 SR-DEVD-FMK probe (SR-FLICA caspase 3/7 assay kit; ImmunoChemistry Technologies LLC), and caspase 8 FAM-LETD-FMK probe (FAM-FLICA caspase 8 assay kit; ImmunoChemistry Technologies LLC). FACS was performed on a BD Aria and flow cytometry acquisition on a BD LSRiL.

**Isolation and culture of primary human CD4<sup>+</sup> T cells.** Healthy donor blood prepared as a buffy coat was obtained from Etablissement Français du Sang (EFS) (agreement with Institut Pasteur C CPSL UNT, 15/EFS/023). The blood was overlaid on Ficoll (EuroBio) at a ratio of 2:1 (vol/vol) blood to Ficoll and centrifuged at 1,800 rpm for 30 min at minimum acceleration/deceleration to obtain PBMCs. CD4<sup>+</sup> T cells were then purified from the PBMCs by negative selection using a StemCell EasySep human CD4<sup>+</sup> T cell isolation kit. Cells were counted and cultured in RPMI 1640 containing Glutamax (ThermoFisher), 10% fetal bovine serum (FBS), penicillin-streptomycin (ThermoFisher; 100 U/ml), and IL-2 (Miltenyi; 100 U/ml) (referred to below as culture medium) at 10<sup>6</sup> cells/ml at 37°C in a 5% CO<sub>2</sub> humidified incubator. The cells were activated with soluble anti-CD3 (clone UCHT-1; Biolegend) for 5 days prior to infection or analysis as previously described (44).

**Isolation and culture of primary infected macaque CD4<sup>+</sup> T cells.** Macaque splenic CD4<sup>+</sup> T cells were obtained from cynomolgus macaques (*Macaca fascicularis*) that were imported from Mauritius and housed at Commissariat à l'Energie Atomique et aux Energies Alternatives (CEA), Fontenay-aux-Roses, France, in compliance with the Standards for Human Care and Use of Laboratory Animals (assurance number A5826-01). The animals were part of the pVISCANTI study, which received ethics approval under the number APAFIS#2453-2015102713323361 v2. They were infected intravenously with 1,000 animal infectious dose 50 (AID<sub>50</sub>) of SIV<sub>mac251</sub>, and sacrificed at a study endpoint, at which time spleen samples were obtained. The macaque splenic CD4<sup>+</sup> T cells were purified by mechanical disruption of a spleen sample in RPMI medium using a GengleMACS dissociator (Miltenyi) followed by cell overlay over Ficoll (EuroBio) (diluted with PBS to 90% prior to use) and centrifugation at 350 × g for 20 min. The cells were then subjected to red cell lysis and then to CD4<sup>+</sup> T cell negative selection using a CD4<sup>+</sup> T cell negative-selection kit (Miltenyi). The cells were cultured in culture medium overnight prior to incubation with peptides. Viral spread from *in vivo*-infected cells was monitored by enzyme-linked immunosorbent assay (ELISA) quantification of p27 (XpressBio) levels on culture supernatants.

**Infection and peptide treatment of primary CD4<sup>+</sup> T cells.** Activated CD4<sup>+</sup> T cells were infected with either Bal (2.9 ng/ml p24), BX08 (21 ng/ml p24), DH12 (3 ng/ml p24), 132w (6.3 ng/ml p24), NL4.3 (7 ng/ml p24), VSVG-pseudotyped *Δenv-Δnef*-GFP (7 ng/ml p24) virus or SIV<sub>mac251</sub> (36.7 ng/ml p27) by centrifuging at 1,200 × g for 1 h at room temperature and then incubating for 1 h at 37°C in a humidified 5% CO<sub>2</sub> incubator. The cells were then washed once with phosphate-buffered saline (PBS), incubated in culture medium, and treated with peptides at 6 μM concentration unless otherwise indicated. Cell death and infection were measured on day 3 postinfection unless otherwise stated. Cell death was evaluated using flow cytometry (LIVE/DEAD violet viability dye; ThermoFisher), and infection was evaluated by either flow cytometry (intracellular p24 staining) or p24/p27 ELISA (XpressBio).

**Real-time flow cytometry.** Cells were washed once with PBS and incubated in annexin buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl<sub>2</sub>, pH 7.4) in the presence of annexin V-FITC (Biolegend) at a concentration of 10<sup>6</sup> cells/ml for 15 min. 7-AAD (Biolegend) was added for the last 5 min of incubation. The cells were then directly passed through the flow cytometer to acquire fluorescence versus time without washing. Alternatively, cells were stained with APG-2 AM (Abcam), a fluorescent K<sup>+</sup> indicator, at a final concentration of 1 μM in plain RPMI 1640 medium for 30 min at room temperature, washed twice with plain RPMI 1640, resuspended in PBS at 10<sup>6</sup> cells/ml, and incubated with 7-AAD (Biolegend) for 5 min before acquisition. To measure mitochondrial membrane potential, cells were stained with JC-1 indicator (ThermoFisher) at a final concentration of 2 μM in PBS at 37°C for 15 min, washed once, and analyzed on a flow cytometer; 6 μM peptides was added after 2 min of baseline acquisition, and acquisition continued for an additional 28 min.

**Caspase 2 inhibition.** *In vitro*-activated CD4<sup>+</sup> T cells were pretreated with FAM-VDVAD-FMK caspase 2 inhibitor (ImmunoChemistry Technologies LLC) according to the manufacturer's instructions for 30 min at 37°C and then pulsed with 6 μM RT53 or RK16 for 5 h. Cell death and caspase 2 activity were evaluated by flow cytometry.

**Immunofluorescence microscopy.** Activated CD4<sup>+</sup> T cells were incubated with 6 μM RT53-rhodamine (rhodamine-RQIKIWFQNRMRMKWKKAKLNAEKLKDFKIRLQYFARGLQYIRQLRLALQGKT) or RK16-rhodamine (rhodamine-RQIKIWFQNRMRMKWKK) (Proteogenix) for 2 h. The cells were then washed twice with PBS, immobilized on polylysine-coated coverslips, fixed with 4% paraformaldehyde for 10 min at room temperature, neutralized with 50 mM NH<sub>4</sub>Cl for 10 min at room temperature, and washed twice with PBS. The cells were then permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. All antibody incubations were performed in 1% bovine serum albumin (BSA) in PBS. Intracellular



staining for AAC-11 was performed with anti-AAC-11 antibody (Abcam; ab65836) at 1/200 dilution for 1 h at room temperature, followed by anti-rabbit IgG-A647 secondary antibody (Life Technologies; A31573) at 1/400 dilution for 45 min at room temperature. The coverslips were then washed, stained with DAPI (4',6-diamidino-2-phenylindole) for 15 min at room temperature, and mounted using Fluoromount G (ThermoFisher) mounting medium.

For analyses of CCR5 distribution, activated CD4<sup>+</sup> T cells were incubated with 6  $\mu$ M RT53 or RK16 (Proteogenix) for 2 h. The cells were then washed twice with PBS, immobilized on polylysine-coated coverslips, fixed with 4% paraformaldehyde for 10 min at room temperature, neutralized with 50 mM NH<sub>4</sub>Cl for 10 min at room temperature, and washed twice with PBS. All antibody incubations and washes were performed in 1% BSA in PBS. The cells were incubated with primary mouse anti-CD4 antibody (clone OKT4; Tonbo Biosciences; 70-0048-U100) and rabbit polyclonal anti-CCR5 (Abcam; ab7346) at 1/100 dilution for 1 h at room temperature, washed, and incubated with secondary antibodies (anti-mouse IgG-A488 [Life Technologies; A11029] and anti-rabbit IgG-A647 [Life Technologies; A31573]) at 1/1,000 dilution for 30 min at room temperature. The coverslips were then washed once in PBS and mounted using Fluoromount G (ThermoFisher) mounting medium.

**Measurements of cellular metabolism.** The OCR and ECAR were measured on a Seahorse XF96 analyzer using a Seahorse XF Cell Mito stress test kit (Agilent). Briefly, activated CD4<sup>+</sup> T cells were incubated with 6  $\mu$ M RT53 or RK16 for 4 h at 37°C. The cells were then counted, washed in Seahorse XF medium (Agilent Seahorse XF base medium with 2 mM Glutamax [Agilent; 102365-100]) containing 10 mM glucose (Sigma), 2 mM sodium pyruvate (Life Technologies), and adjusted to pH 7.4. An equal number of live cells were seeded at  $2 \times 10^5$  cells per well in XF96 V3 P5 plates (Seahorse Bioscience) precoated with 0.5 mg/ml Corning Cell-Tack cell and tissue adhesive (Corning; 354240) and incubated for a minimum of 30 min in a CO<sub>2</sub>-free 37°C incubator prior to acquisition. The following drugs were placed at injection ports: Seahorse XF medium (port A), 2.5  $\mu$ M oligomycin (port B), 0.9  $\mu$ M FCCP (port C), and 1  $\mu$ M rotenone and 1  $\mu$ M antimycin A (port D).

**Quantitative RT-PCR. (i) Evaluating the expression of AAC-11 and other genes in CD4<sup>+</sup> T cell memory subsets.** The expression levels of AAC-11 and other genes on CD4<sup>+</sup> T cell subsets were analyzed in a previous study (7, data set [doi:10.17632/vfj3r27gnf.1]). Briefly, the total RNA from  $5 \times 10^6$  cells was extracted using an RNA trace kit (Macherey-Nagel), treated with DNase, reverse transcribed using reverse transcription master mix (Fluidigm), preamplified using PreAmp master mix (Fluidigm), and treated with exonuclease I (New England Biolabs). Samples were then mixed with SsoFast EvaGreen Supermix with Low ROX (Bio-Rad), DNA binding dye (Fluidigm), and assay mix (assay loading reagent [Fluidigm] and Delta Gene primers [Fluidigm]). The expression levels were read on a Biomark HQ system (Fluidigm). The expression levels of BECN1 were used for normalization. Gene expression values were plotted as  $2^{-\Delta\Delta CT}$ .

**(ii) Evaluating the expression of viral gene products.** Cells were collected by centrifugation, and the dry pellet was stored at  $-80^\circ\text{C}$  until DNA extraction. DNA was extracted using a NucleoSpin tissue kit (Macherey-Nagel). Real-time PCR (RT-PCR) to quantify total and integrated HIV-1 DNA was performed as described previously (64) using TaqMan universal PCR master mix (ThermoFisher). Briefly, total viral DNA was quantified using the following primers and probe: CTTTCGCTTCAAGTCCCTGTT (forward), AGATCCCTCAGACCCCTTTAGTCA (reverse), and FAM-TGGAAAATCTTAGCAGTGGCGCCC-black hole quencher 1 (BHQ1) (probe). The 8E5 cell line containing a single viral copy per cell was used as a standard. Integrated viral DNA was quantified by first preamplifying the DNA using AccuTaq LA DNA polymerase (Sigma) and the following primers: AGCCTCCCGAGTAGCTGGGA (FirstAluF), TTACAGGCATG AGCCACCG (FirstAluR), and CAATATCATAACGCGAGAGTGGCGCTTCAGCAAG (NY1R). A second DNA amplification round was performed with TaqMan universal PCR master mix (ThermoFisher) using the following primers: AATAAAGCTTGCCTTGAGTGCTC (NY2F), CAATATCATAACGCGAGAGTGC (NY2R), and FAM-AGTGTGTGCCCTGCTGTGTGACTC-6-carboxytetramethylrhodamine (TAMRA) (NY2ALU probe). HeLa cells containing HIV-1 integrated DNA were used as a standard (64). The results were normalized to nanograms of actin using a human DNA standard (Sigma). The primers and probe used for quantification of actin were TGCATGAGAAAACGCCAGTAA (forward), ATGGTCGCTGTTCACCAA (reverse), and FAM-TGACAGAGTACCAAAATGCTGCACAGAA-TAMRA (probe).

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism software. Linked parametric or nonparametric one-way analysis of variance (ANOVA) or two-way ANOVA was used, depending on the experiment. Dunnett's, Dunn's, or Holm-Sidak's multiple-comparison tests were used for *post hoc* analysis.

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A.M., J.C.V.-C., A.D., V.M., and A.E. performed experiments; A.M., J.C.V.-C., A.D., V.M.,

S.V., and A.S.-C. analyzed the data; J.-L.P. and C.P. provided key reagents; J.-L.P. and A.S.-C. conceived the study; A.M., A.D., J.C.V.-C., and A.S.-C. designed the experiments; A.S.-C. supervised the study; A.M. and A.S.-C. drafted the article; and we all critically reviewed the manuscript.

A.M., A.D., J.-L.P., and A.S.-C. are listed as inventors in a patent application submitted by INSERM and Institut Pasteur partially based on the results described here. J.-L.P. is listed as inventor in a patent application related to the use of AAC-11 peptides in the treatment of cancer.

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## FRENCH SUBSTANTIAL SUMMARY

Actuellement, il n'existe toujours pas de thérapie curative contre le virus de l'immunodéficience humaine 1 (VIH-1), mais les traitements antirétroviraux actuels permettent de contrôler l'infection et empêcher la progression vers le syndrome d'immunodéficience acquise (SIDA). Toutefois, bien que la suppression de la réplication du VIH-1 soit possible grâce aux traitements antirétroviraux, leurs effets secondaires, la résistance, la stigmatisation et le coût sont autant d'éléments qui soulignent la nécessité de poursuivre le développement de stratégies thérapeutiques pour le traitement du VIH-1. De plus, le traitement antirétroviral seul ne permet pas d'éradiquer le VIH-1, car même après des années de traitement intensif et continu, la propagation du virus reprend en quelques semaines après l'arrêt du traitement, sauf dans des cas exceptionnels. Le génome du VIH-1 intact et compétent à la réplication est capable de persister pendant des décennies dans des cellules à longue durée qui sont à l'origine du rebond viral rapide lors de l'interruption du traitement. Cibler ces réservoirs est actuellement l'une des principales priorités dans le domaine de la recherche sur le VIH-1.

Plusieurs études ont montré que les cellules T CD4<sup>+</sup> mémoires (mémoire centrale, T<sub>CM</sub>; mémoire transitoire, T<sub>TM</sub>; mémoire effectrice, T<sub>EM</sub>) sont les cellules principalement infectées chez les individus sous traitement antirétroviral. Malgré la faible fréquence des cellules T CD4<sup>+</sup> naïves (T<sub>N</sub>) infectées, cette sous-population représente un important réservoir viral après un traitement rétroviral prolongé, en raison de leur très longue durée de vie. De même, les cellules T CD4<sup>+</sup> mémoires semblables à des cellules souches (T<sub>SCM</sub>), bien qu'elles ne représentent qu'une fraction mineure des cellules T CD4<sup>+</sup> totales, peuvent également représenter un réservoir important du VIH-1 en raison de leur longue durée de vie. Il est intéressant de souligner que malgré le fait que les différentes sous-populations de T CD4<sup>+</sup> puissent héberger le génome viral, ces sous-populations ne présentent pas la même susceptibilité au VIH-1. Les cellules les moins différenciées, T<sub>N</sub>, sont relativement résistantes, alors que les cellules plus différenciées, T<sub>EM</sub>, sont très sensibles à l'infection. Cela suggère qu'au cours de leur processus de différenciation, les cellules T CD4<sup>+</sup> subissent des changements intracellulaires qui ont un impact décisif sur l'infection par le VIH-1. La différenciation de ces cellules est caractérisée par des changements drastiques dans le métabolisme cellulaire pour permettre la mise en place des fonctions effectrices ainsi que leur expansion. Des travaux récents de l'équipe d'accueil ainsi que d'autres laboratoires ont montré que le métabolisme cellulaire joue un rôle clé dans la modulation de la permissivité des T CD4<sup>+</sup> à l'infection par le VIH-1.

En effet, une notion relativement nouvelle en immunologie postule que l'activité métabolique et l'activation des cellules immunitaires sont étroitement liées. L'immunométabolisme, c'est-à-dire l'étude du dialogue entre les voies bioénergétiques et les fonctions des cellules immunitaires, est de plus en plus reconnu comme un domaine scientifique important et prometteur. Jusqu'à présent, l'immunologie a mis l'accent sur l'étude de la façon dont les cellules immunitaires reçoivent des signaux afin de reconnaître les antigènes et de coordonner la réponse immunitaire. Cependant, jusqu'à récemment, la majorité des recherches se concentraient sur les aspects moléculaires et négligeaient essentiellement toute pertinence pour le métabolisme dans ce domaine. Parallèlement, la recherche sur le cancer s'est concentrée sur le métabolisme depuis de nombreuses années, car la croissance incontrôlée des cellules



cancéreuses révélait des faiblesses métaboliques pouvant être exploitées. L'attention portée à l'immunométabolisme s'est accrue avec l'identification d'un parallèle métabolique entre les deux domaines, fondé sur ce qui est maintenant appelé le "l'effet Warburg", qui consiste en une augmentation du taux de consommation du glucose avec une production préférentielle de lactate contrairement au pyruvate, même en présence d'oxygène, pour soutenir métaboliquement leur processus d'activation. Depuis lors, le domaine de l'immunométabolisme a connu une croissance explosive et s'est étendue à d'autres domaines que la cancérologie.

Le laboratoire d'accueil a montré que la hiérarchie des activités métaboliques (phosphorylation oxydative et glycolyse) des sous-populations de cellules T CD4+ correspondait à la hiérarchie de susceptibilité à l'infection par le VIH-1 de ces mêmes sous-populations ( $T_{EM} > T_{TM} \geq T_{CM} > T_N$ ). Les  $T_N$  qui présentaient une forte résistance à l'infection, montraient une capacité métabolique la plus faible parmi les différentes sous-populations. Les  $T_{EM}$  cependant, présentaient un taux de glycolyse, une respiration mitochondriale et un pourcentage d'infection très élevé. En outre, les cellules T CD4+ purifiées et infectées par le VIH-1 *in vitro* possédaient des activités métaboliques plus élevées que les cellules T CD4+ qui ont été exposées au virus mais qui n'ont pas donné lieu à une infection. De plus, au sein d'une même sous-population, les cellules ayant une activité métabolique élevée étaient permissives à l'infection tandis que les cellules à faible activité métabolique étaient fortement résistantes à l'infection. Dans l'ensemble, ces résultats ont montré que le VIH-1 infecte sélectivement les cellules T CD4+ présentant des niveaux élevés de phosphorylation oxydative et de glycolyse. Cela suggère que le métabolisme cellulaire est important pour le cycle de réplication du VIH-1 et que le ciblage du métabolisme cellulaire pourrait représenter une approche thérapeutique pour la guérison du VIH-1.

La réplication virale et la mobilisation d'une réponse immunitaire ont en commun de dépendre des nutriments, des intermédiaires métaboliques et de l'énergie disponibles dans la cellule. Comme elles sont le siège de la réplication virale, le virus va détourner une partie importante de la machinerie et des intermédiaires métaboliques des cellules T CD4+, initialement mis en place pour développer une réponse immunitaire efficace. De plus, ce détournement peut altérer le métabolisme cellulaire et mettre en péril la réponse immunitaire. Par ailleurs, comme elle fournit à la cellule des ressources métaboliques supplémentaires, cette reprogrammation du métabolisme cellulaire de la cellule hôte peut être très favorable à la réplication virale. Par exemple des sous-populations distinctes de cellules T CD4+ utilisent des voies métaboliques différentes pour soutenir leur fonction, les cellules  $T_{H1}$ ,  $T_{H2}$  et  $T_{H17}$  utilisant la glycolyse et les cellules  $T_{REG}$  préférant l'oxydation des acides gras. Nous proposons donc que le statut métabolique des cellules T CD4+ est un des facteurs déterminants de leur sensibilité au VIH-1.

Notre premier objectif était de vérifier cette hypothèse en employant différents inhibiteurs métaboliques qui interféreraient avec les environnements métaboliques intrinsèques ou induits par le virus qui favorisent l'infection. Grâce à la recherche sur le cancer et les maladies métaboliques, il existe déjà de nombreux médicaments présentant un potentiel pour être utilisé, en ciblant différentes voies métaboliques.

L'inhibition de la glycolyse à l'aide d'un analogue de glucose, le 2-désoxyglucose (2-DG), ou avec l'agent alkylant et analogue de l'acide pyruvique, le 3-bromopyruvate, a réduit l'infection

par le VIH-1, suggérant l'importance de la glycolyse pour la réplication virale. L'utilisation d'un inhibiteur du transport du pyruvate, le Zaprinst, a également montré que l'utilisation de métabolites de la glycolyse dans la respiration mitochondriale est cruciale pour l'infection virale. Cependant, l'inhibition de la glutaminolyse n'a pas modifié les niveaux d'infection, ce qui contredit les résultats obtenus par d'autres équipes et la nôtre en utilisant un virus VIH-1 à cycle unique. Cela a conduit à l'hypothèse que la glutaminolyse interfère avec les premières étapes de la réplication virale et que l'effet d'une telle inhibition est perdue avec un virus sauvage présentant un cycle de réplication complet, au moins en employant des doses non cytotoxiques de ces inhibiteurs. L'utilisation de l'Etomoxir, un inhibiteur de la bêta-oxydation des acides gras, n'a pas modifié la réplication du virus. Cependant, l'inhibition de la synthèse des acides gras avec TOFA, C75 et l'agent anti-obésité Orlistat, a induit le blocage de l'infection.

Nous avons donc conclu que la glycolyse, la respiration mitochondriale ainsi que la synthèse d'acides gras sont des voies importantes pour l'infection VIH-1 et qu'elles peuvent constituer de potentielles cibles dans les stratégies de lutte contre l'infection.

La glycolyse élevée peut favoriser les premières étapes de la réplication virale en améliorant la biosynthèse des désoxyribonucléotides triphosphates (dNTPs) nécessaires pour la transcription réverse de l'ARN virale. La glycolyse aérobie génère aussi la forme oxydée du nicotinamide adénine dinucléotide (NAD<sup>+</sup>), porteur redox intervenant dans nombreux processus métaboliques. Le NAD<sup>+</sup> sert également d'analogue de nucléotide dans la ligature de l'ADN et la synthèse de la coiffe de l'ARN messager. De plus, la conversion du pyruvate en acétyl-CoA dans les mitochondries par le cycle de Krebs favorise l'acétylation de la  $\alpha$ -tubuline et la stabilisation des microtubules cooptés pour le transport cytoplasmique du VIH-1. D'autre part, l'acétyl-CoA contribue à l'acétylation des histones et sa production peut moduler la transcription du VIH-1. De plus, l'acétyl-CoA est un précurseur clé de la synthèse *de novo* du cholestérol qui est important pour la formation des radeaux lipidiques essentiels à l'entrée du virus, au trafic des protéines virales, à l'assemblage et au bourgeonnement de virions.

Nous avons ensuite voulu étudier les facteurs susceptibles de modifier la sensibilité des lymphocytes T CD4<sup>+</sup> au VIH-1 et de rendre hautement permissives des sous-populations censées être résistantes à l'infection pendant la phase aiguë de l'infection. Cette phase initiale correspond très probablement au moment de l'ensemencement du réservoir, étant donné qu'un traitement antirétroviral précoce n'empêche pas l'établissement de ce réservoir. Au cours de cette phase très précoce, plusieurs cytokines jouant un rôle antiviral, pro-inflammatoire ou immunosuppresseur sont connues pour être sécrétées en quantités accrues. Nous avons émis l'hypothèse que ces cytokines, dont certaines sont associées à des taux d'infection plus élevés, pourraient également avoir un impact sur le métabolisme cellulaire des cellules T CD4<sup>+</sup>. Nous montrons que les cytokines pro-inflammatoires, l'interleukine (IL)-2, IL-7 et IL-15, qui jouent un rôle dans l'homéostasie T, connues et confirmées dans nos expériences pour augmenter l'infection par le VIH-1, favorisent la glycolyse et la phosphorylation oxydative. À l'inverse, l'IL-21, qui a entraîné une diminution de l'infection par le VIH-1, a reprogrammé les cellules vers une légère augmentation de la glycolyse, mais aussi une diminution significative de la phosphorylation oxydative. L'ajout de l'IL-10 a contrecarré l'augmentation de l'infection causée par IL-2 et IL-7. De manière plus intéressante, l'IL-10 a également empêché l'accroissement de l'activité métabolique habituellement induite par l'IL-2 et l'IL-7.

Nos travaux donnent un aperçu du rôle potentiel des cytokines dans la définition de la dynamique de l'ensemencement des réservoirs pendant la phase aigüe. Les résultats obtenus suggèrent que la reprogrammation métabolique peut être l'un des moyens par lesquels les cytokines influencent la susceptibilité à l'infection par le VIH-1. Nous avons montré que trois voies métaboliques - la glycolyse, la phosphorylation oxydative et la synthèse des acides gras - sont cruciales pour l'infection des cellules CD4+ par le VIH-1. Nos résultats ont aussi suscité l'intérêt pour un potentiel thérapeutique de ciblage du métabolisme cellulaire dans la lutte contre l'infection. Nous avons émis l'hypothèse que le VIH-1 dépend du métabolisme accru et qu'en reprogrammant l'activité métabolique basale des lymphocytes T CD4+, nous pourrions rétablir un environnement inadéquat pour la réplication du virus. En plus, en raison de leurs besoins métaboliques élevés, les cellules infectées (ou les cellules très sensibles à l'infection) peuvent également être particulièrement sensibles à des concentrations sous-optimales de molécules qui interfèrent avec les processus métaboliques, ce qui pourrait conduire à l'élimination spécifique de ces cellules sans affecter les cellules T CD4+ non infectées, comme cela a été montré pour les cellules cancéreuses par rapport aux cellules saines pour certaines tumeurs. Enfin, l'utilisation d'inhibiteurs métaboliques en combinaison avec d'autres stratégies curatives, comme l'approche "*shock and kill*", pourrait s'avérer extrêmement puissante pour éradiquer le réservoir du VIH-1.