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# AAC-11 derived peptides antagonize the survival and infection of HIV-1 susceptible CD4+T cells and macrophages

Anastassia Mikhailova

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Thèse de doctorat  
de l'Université Sorbonne Paris Cité  
Préparée à l'Université Paris Diderot  
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*Unité HIV, Inflammation et Persistance, Institut Pasteur, Paris*

# **AAC-11 derived peptides antagonize the survival and infection of HIV-1 susceptible CD4+ T cells and macrophages**

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Thèse de doctorat de Virologie

Dirigée par Dr. Asier Sáez-Cirión

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## ABSTRACT

Despite its discovery more than 30 years ago, HIV epidemic remains one of the global health challenges of today. Among many important questions remaining is how does HIV-1 successfully establish infection and persist in its reservoirs despite viral cytotoxic effect observed both *in vitro* and *in vivo*? This work shows that HIV-1 relies on AAC-11 anti-apoptotic pathway for the establishment of productive infection in CD4+ T cells and macrophages, primary targets of HIV-1. We observed the expression of AAC-11 to increase with progressive CD4+ memory T cell differentiation and to associate with the expression of cell cycle, activation and metabolism genes, known factors for HIV-1 susceptibility. Antagonism of AAC-11 survival pathway with the peptides derived from its sequence lead to cell death of HIV-1 susceptible target cells and resistance of surviving cells to infection. The peptides caused preferential elimination of effector and transitional CD4+ T cell memory subsets as well as highly activated and metabolically active cells, and their activity was at least in part dependent on caspase 2 activation. Thus, our results provide a proof of concept that the selective targeting of the survival pathways used by HIV-1 is a possible approach to antagonize the seeding of HIV-1 CD4+ T cell and macrophage reservoirs.

### **Keywords:**

Anti-apoptotic clone 11, human immunodeficiency virus, CD4+ T cell, cell death, immuno-metabolism, HIV-1 dependency factors

## RÉSUMÉ

Malgré plus de 30 ans de recherche intensive depuis le début de l'épidémie, le virus de l'immunodéficience humaine (VIH) reste l'un des plus gros problèmes de santé publique. L'une des problématiques les plus importantes empêchant le progrès vers la résolution de combat avec cette maladie reste de comprendre comment le VIH de type 1 établit l'infection et la persistance dans ses réservoirs. Et ce, malgré l'effet cytotoxique du virus qui est observé in vitro et in vivo. Ce travail montre que le VIH-1 dépend de la voie de signalisation anti-apoptotique d'AAC-11 pour établir l'infection dans les lymphocytes CD4<sup>+</sup> T et les macrophages qui sont les cibles importantes du VIH-1. Nous observons que l'expression d'AAC-11 augmente avec la différenciation des lymphocytes CD4<sup>+</sup> T mémoires. Nous avons également remarqué que l'expression d'AAC-11 est associée avec l'expression des gènes du cycle cellulaire, de l'activation et du métabolisme, des facteurs qui jouent un rôle dans la susceptibilité du VIH-1. Nous avons modulé la voie de signalisation d'AAC-11 avec l'antagonisme provenant des peptides dérivés de sa séquence et observé la mort cellulaire des cellules susceptibles à l'infection. Nous avons également observé que les cellules restantes étaient résistantes à l'infection. Les peptides ont éliminé préférentiellement les cellules effectrices et transitionnelles mémoires CD4<sup>+</sup> T, ainsi que les cellules activées et métaboliquement actives. Leur activité était en partie dépendante de l'activation de caspase-2. Par conséquent, nos résultats présentent une preuve de concept que le ciblage des voies de survie cellulaires utilisées par le VIH est une approche plausible pour impacter négativement l'ensemencement du réservoir de VIH, dont font partie les cellules CD4<sup>+</sup> T et macrophages.

### **Mots-clés:**

Virus de l'immunodéficience humaine, mort cellulaire, lymphocyte CD4<sup>+</sup> T, macrophage

Human immunodeficiency virus, cell death, CD4<sup>+</sup> T lymphocyte, macrophage

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## LIST OF ABBREVIATIONS

AAC-11	anti-apoptotic clone 11
Ag	antigen
AMPK	5'-AMP activated protein kinase
ASC	apoptosis-associated speck-like protein containing C-terminal CARD
ATM	<i>ataxia telangiectasia mutated</i>
ATP1A1	Na <sup>+</sup> /K <sup>+</sup> ATPase subunit $\alpha$ isoform 1
ATP1A2	Na <sup>+</sup> /K <sup>+</sup> ATPase subunit $\alpha$ isoform 2
ATP1A3	Na <sup>+</sup> /K <sup>+</sup> ATPase subunit $\alpha$ isoform 3
ATP1A4	Na <sup>+</sup> /K <sup>+</sup> ATPase subunit $\alpha$ isoform 4
ATP1B1	Na <sup>+</sup> /K <sup>+</sup> ATPase subunit $\beta$ isoform 1
ATP1B2	Na <sup>+</sup> /K <sup>+</sup> ATPase subunit $\beta$ isoform 2
ATP1B3	Na <sup>+</sup> /K <sup>+</sup> ATPase subunit $\beta$ isoform 3
ATP5B	ATP synthase subunit $\beta$
BAD	BCL2 antagonist of cell death
BAK	BCL2 antagonist/killer
BAX	BCL2 associated X protein
BID	truncated BH3-interacting domain death
BIM	BCL2 interacting mediator of cell death
CA	capsid
CAMKII	calcium/calmodulin dependent kinase II
CARD	caspase activation and recruitment domain
cART	combination anti-retroviral therapy
CCR7	C-C chemokine receptor type 7



CD27	cluster of differentiation 27
CD4	cluster of differentiation 4
CD45R0	cluster of differentiation 45 R0
CD45RA	cluster of differentiation 45 RA
CDK2	cyclin dependent kinase 2
CDK9	cyclin dependent kinase 9
CG	cardiac glycoside
CNS	central nervous system
CPSF6	cleavage and polyadenulation specificity factor 6
CRAC	Ca <sup>2+</sup> release-activated Ca <sup>2+</sup> channel
CTL	cytotoxic T cell response
CycT1	cyclin T1
CypA	cyclophilin A
DAMP	damage-associated molecular pattern
DC	dendritic cell
DNA-PK	DNA-dependent protein kinase
EDTA	ethylenediaminetetraacetic acid
ENO1	$\alpha$ -enolase
Env	envelope protein
ER	endoplasmic reticulum
ESCRT	endosomal sorting complex required for transport
FAO	fatty acid oxidation
FAS	fatty acid synthesis
FMO	fluorescence minus one
HDF	HIV-1 denependency factor

HDF	HIV-1 dependency factor
HMGA1	High mobility group protein A1
IFN	interferon
IKK	I $\kappa$ B kinase
IMM	inner mitochondrial membrane
IN	integrase
LDHA	L-lactate dehydrogenase $\alpha$ chain
LEDGF/p75	Lens epithelium derived growth factor
LTR	long-terminal repeat
LZ	leucine zipper
MA	matrix
Mdm2	mouse double minute 2
MFI	mean fluorescence intensity
MLKL	mixed lineage kinase domain-like
MOMP	mitochondrial outer membrane permeabilization
MPT	mitochondrial permeability transition
MPTP	mitochondrial permeability transition pore
mTOR	mechanistic target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
NC	nucleocapsid
NFAT	nuclear factor of activated T cells
NF $\kappa$ B	nuclear factor $\kappa$ -light-chain-enhancer of activated B cells
NKA	Na <sup>+</sup> /K <sup>+</sup> ATPase
NPC	nuclear pore complex

NRTI	nucleoside analog reverse transcriptase inhibitor
OMM	outer mitochondrial membrane
OXPPOS	oxidative phosphorylation
P-TEFb	Positive transcriptional elongation factor b
PAK1	serine/threonine-protein kinase PAK1
PAMP	pathogen-associated molecular pattern
PARP	Poly(ADP-ribose) polymerase
PBMCs	peripheral blood mononuclear cells
PIC	pre-integration complex
PIDD	p53-induced protein with a death domain DD
PM	plasma membrane
PPP	pentose phosphate pathway
PRR	pattern recognition receptor
PtdIns(4,5)P <sub>2</sub>	phosphatidylinositol-4,5-bisphosphate
PTPC	permeability transition pore complex
PUMA	p53-upregulated mediator of apoptosis
RAG	recombination activating gene
RAIDD	receptor-interacting protein-associated ICH-1/CED-3 homologous protein with a DD
Rev	regulator of expression of viral proteins
RF	restriction factor
RF	restriction factor
RIPK1	receptor interacting protein kinase 1
RIPK3	receptor interacting protein kinase 3
RT	reverse transcription/reverse transcriptase
Sp1	specificity protein 1

$t_{1/2}$	half-life
TAR	transactivation response element
tBID	truncated BID
TBP	TATA binding protein
Tcm	central memory CD4+ T cells
TCR	T cell receptor
Te	effector CD4+ T cells
Tem	effector memory CD4+ T cells
TF	transcription factor
Tfh	T follicular helper cell
Tn	naïve CD4+ T cells
TRPM4	transient receptor potential cation channel subfamily M member 4
Tscm	stem cell-like memory CD4+ T cells
Ttm	transitional memory CD4+ T cells
Vpr	viral protein R

# INTRODUCTION

## PREFACE

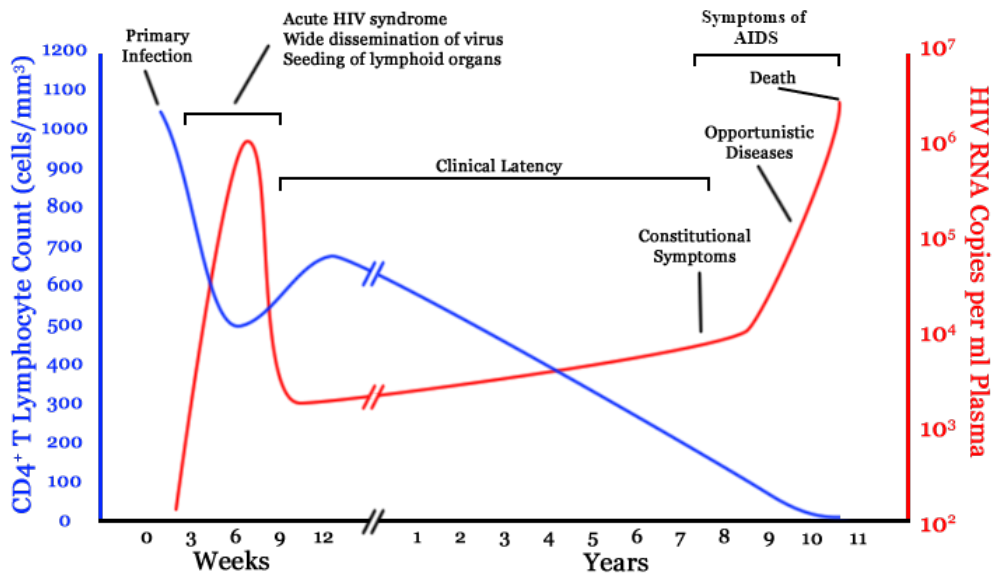
Worldwide Human immunodeficiency virus (HIV) epidemic still remains a major global health challenge, despite the existence of combination anti-retroviral therapy (cART). In 2017, the global population of people living with HIV was estimated to be 36.9 million, with 0.94 million new infections and 1 million acquired immunodeficiency syndrome (AIDS)-related deaths that year (UNAIDS). The number of new infections is steadily declining in some areas of the world (strongest decrease seen in Eastern, southern Africa, Western and central Europe as well as North America), while remaining steady (Latin America) or even increasing (Eastern Europe) in others (UNAIDS). Socio-economic barriers undoubtedly present a major challenge to control of the epidemic. It is, therefore, imperative to continue research on the immunopathology of HIV infection with the goal to design vaccine and curative strategies, which do not exist today.

HIV has two subtypes, HIV-1 and HIV-2, both of which cause AIDS. HIV-2 causes milder symptoms with most infected individuals not progressing to AIDS. HIV-2 virus remains largely restricted to West Africa and its prevalence rates are declining due to lower transmission rates (Sharp and Hahn, 2011). Infection with HIV-2 will not be considered here. HIV-1 consists of four genetically distinct groups: M (major), O (outlier), N (non-M, non-O), and P (Garcia-Tellez et al., 2016). Group O represents less than 1% of global infections and is restricted to Central West Africa (Cameroon, Gabon and neighbouring countries). Cases from groups N and P are even more restricted with only few cases identified for each of these groups (Sharp and Hahn, 2011). The pandemic group M is further subdivided into many subtypes (A, B, C, D, F, G, H, J, K) with distinct geographical distribution. Dominant subtype in Americas, Western Europe and Australia is subtype B. Subtype C is prevalent in Southern Africa and India. In addition, great number of recombinant circulating forms that are a mix of different subtypes exist.

## BRIEF NATURAL HISTORY OF HIV-1 INFECTION

HIV spreads by exposure of mucosal surfaces (sexual transmission, breast feeding) or through direct blood contact (intravenous drug use, transfusion, mother to child transmission during pregnancy and delivery). During sexual transmission, HIV-1 encounters its first target cells in the genital and rectal mucosa (dendritic cells, macrophages, resident CD4+ T cells), which then carry the virus to peripheral lymph nodes and gut associated lymphoid structures for further dissemination to CD4+ T cells in these tissues (Hladik and McElrath, 2008). This process is accompanied by the onset of systemic inflammation that never resolves and serves as a prognostic marker of AIDS.

The first wave of CD4+ T cell infection and massive depletion of this population in lymphoid tissues leads to high viremia associated with flu-like symptoms (fever, headache, rash) and is termed the acute stage of the disease (Figure 1). The risk of HIV transmission is high during this period ("The Stages of HIV Infection Understanding HIV/AIDS," 2018). HIV-1 specific cytotoxic CD8+ T cell (CTL) response develops during this stage of infection somewhat limiting viral replication. This leads to decline of viremia to the viral set point and stabilization of CD4+ T cell count (Borrow et al., 1994; Ndhlovu et al., 2015), which occurs within 3-6 months of infection (McMichael et al., 2010). This stage is termed the chronic phase, is typically asymptomatic and, in the absence of treatment, leads to AIDS in 10 years or less. Risk of transmission still remains at this stage of infection. Viral escape mutations and other viral evasion mechanisms, renders CTL arm of immunity largely ineffective in the control of infection during chronic disease (Borrow et al., 1997; Deng et al., 2015). Natural killer (NK) cell and cytotoxic CD4+ T cell responses also fail to control the virus in majority of patients, although a genetic association between efficient NK cell and CTL responses and HIV control has been reported (McMichael et al., 2010). Similarly, B cell responses are impaired during HIV-1 infection. Production of immune complexes followed by non-neutralizing antibodies is seen early in infection (McMichael et al., 2010). HIV-1 neutralizing antibody responses take years to develop and do not control infection in majority of cases.



**Figure 1. Clinical stages of HIV-1 infection.**

At the end of the chronic phase, CD4+ T cell counts decline further and the viral load drastically rises again marking the onset of AIDS. This stage is marked by the drop of blood CD4+ T cell count to below 200 cells/mm<sup>3</sup> and the onset of opportunistic infections. The transmission chance at this stage is high. The survival rate in the absence of treatment is less than 3 years (“About HIV/AIDS | HIV Basics | Act Against AIDS | CDC,” 2018).

# ORIGIN OF HIV AND NON-HUMAN PRIMATE MODELS OF HIV-1

## INFECTION

HIV-1 and HIV-2 infections of humans have resulted from cross-species transmission events of simian immunodeficiency viruses (SIV) originating from several African primate species (Figure 2). Each of the HIV-1 groups resulted from an independent transmission event. For example, HIV-1 groups M and N originated from West-Central Africa chimpanzees (*Pan troglodytes troglodytes*), in which SIV<sub>cpz</sub> virus causes reduced life span (Garcia-Tellez et al., 2016). These primates, in turn, were infected by simian immunodeficiency virus (SIV) from red-capped mangabey (*Cercocebus torquatus*) and several *Cercopithecus* species. Group P originated from a gorilla SIV (SIV<sub>gor</sub>) transmission event and the origin of Group O is unknown (Sharp and Hahn, 2011). HIV-2 originated from sooty mangabey SIV virus (SIV<sub>smm</sub>), which is non-pathogenic in this natural host (Sharp and Hahn, 2011).

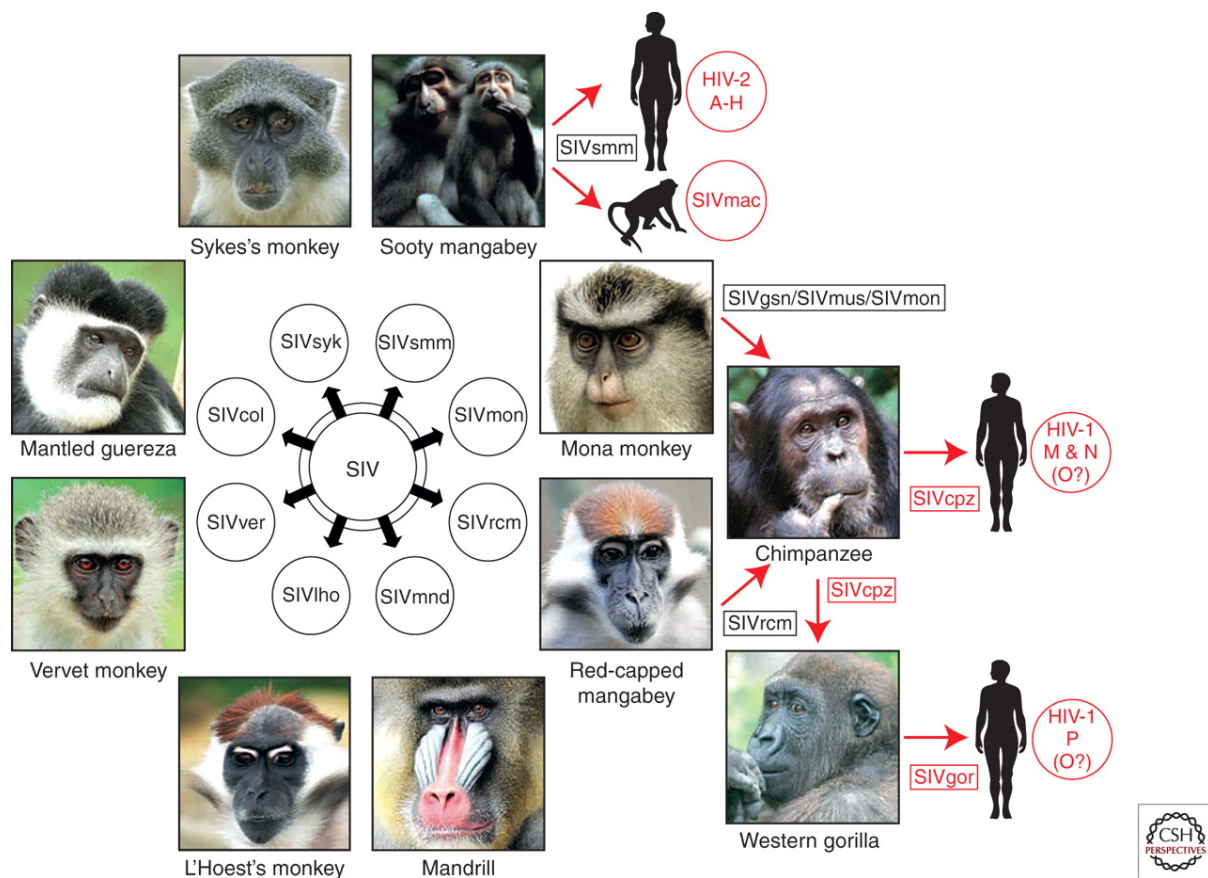


Figure 2. Origins of human AIDS viruses and diversity of simian immunodeficiency viruses.

Adapted from (Sharp and Hahn, 2011)

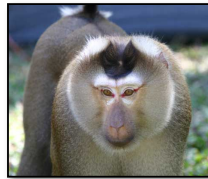


It is not surprising, therefore, that non-human primates (NHP) are widely used as a disease model for HIV-1 infection and AIDS. Importantly, infection of different NHP species of different genetic background with SIV strains of various virulence allows to recapitulate all disease progression profiles found in humans (Figure 3) (Saez-Cirion et al., 2014, p.) (Garcia-Tellez et al., 2016). Three macaque species are mainly used to model disease progression (rapid or slow) to AIDS: cynomolgus macaque (*Macaca nemestrina*), pig-tailed macaque (*Macaca fascicularis*), and rhesus macaque (*Macaca mulatta*) (Figure 3). SIV<sub>mac</sub> viral strains infecting macaques recapitulates HIV-1 infection pathophysiology and mirrors clinical stages of the disease found in humans (Garcia-Tellez et al., 2016) (Sui et al., 2013). Some of the similarities between human and simian disease include: mucosal transmission, tropism for CD4+ T cells and macrophages, clinical stages of the disease and immune activation (Sui et al., 2013).

Fastest progression to AIDS is generally observed upon infection of Indian rhesus macaque with SIV<sub>mac239</sub> strain (Garcia-Tellez et al., 2016). On the other hand, spontaneous viral control resembling natural control of HIV-1 in humans has been demonstrated in rhesus and cynomolgus macaques with specific MHC alleles, similarly to genetic protective associations found in controller humans (Saez-Cirion et al., 2014, p.). Finally, natural SIV infection models in species where the virus does not cause a disease exist: SIV<sub>agm</sub>-infected African green monkeys, SIV<sub>smm</sub>-infected sooty mangabeys and SIV<sub>mnd</sub>-infected mandrills (Jacquelin et al., 2012). The major goal of studying infection of such natural hosts is to identify correlates of protection against disease in these animals and try to induce these mechanisms in animal pre-clinical models and during pathogenesis in humans.



Rhesus macaques  
(*Macaca mulatta*)



Pig-tailed macaques  
(*Macaca nemestrina*)



Cynomolgus macaques  
(*Macaca fascicularis*)

**Non-natural SIV hosts**

**Persistently high viremia**

**Do not control  
inflammation**

**AIDS**

**DISEASE  
PROGRESSION**

*rapid*



*slow*

**VIRAL  
FACTORS**

SIVmac239

SIVmac251

SIVsmm

SIVmac $\Delta$ nef

**HOST  
FACTORS**

Pig-tailed macaques

Rhesus macaques (Indian)

Rhesus macaques (Chinese)

Cynomolgus macaques

**Figure 3. Non-human primate models of HIV-1 caused disease progression found in humans. Courtesy of Passaes CP.**

# HUMAN IMMUNODEFICIENCY VIRUS-1

## *VIRAL PARTICLE*

HIV-1 viral particle is enveloped by a lipid bilayer supported by the matrix (MA) protein and has 5-15 trimers of envelope (Env) glycoproteins embedded into it (Brandenberg et al., 2015). The membrane and MA enclose the viral capsid core, composed of a polymer of capsid protein (CA) arranged in a conical shape (Yamashita and Engelman, 2017). The capsid core contains 2 copies of the 9,230nt long RNA genome, which is decorated with nucleocapsid (NC) proteins. Viral reverse transcriptase (RT), integrase (IN) and other proteins (both viral and host cellular) are also found in the capsid (Yamashita and Engelman, 2017) (Figure 4).

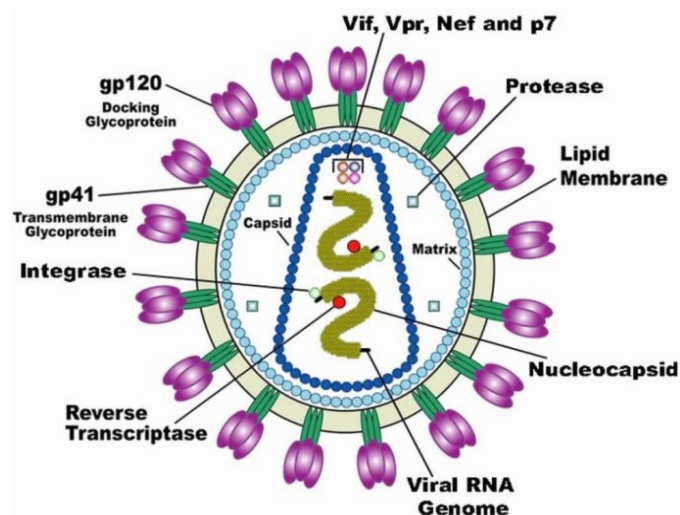
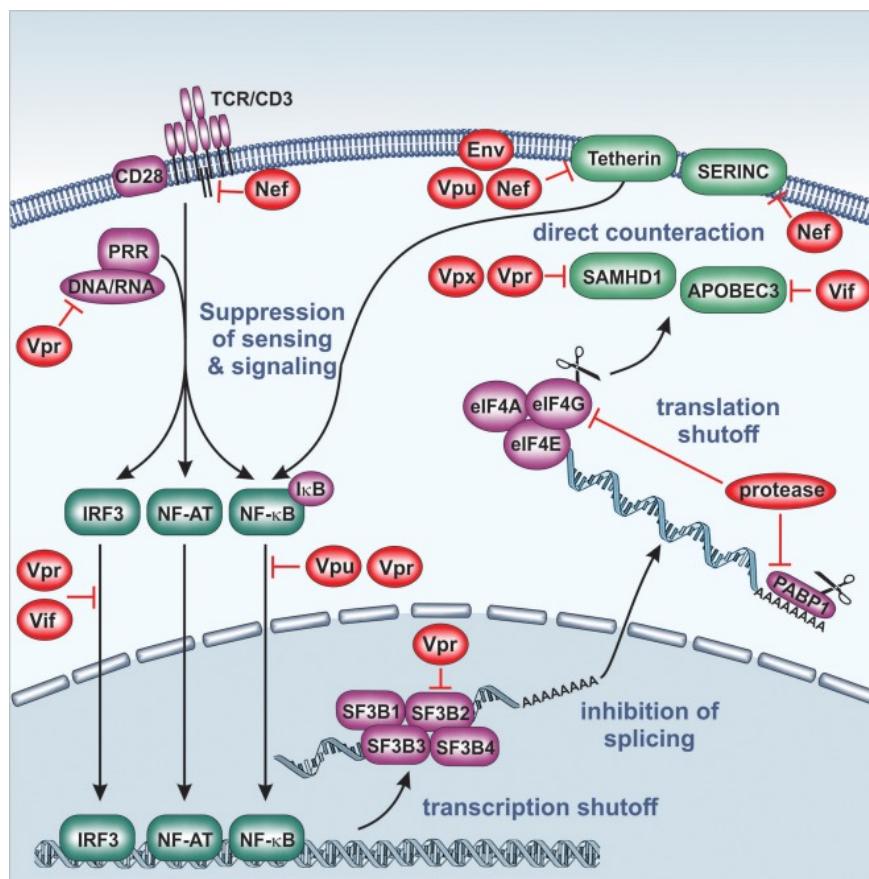


Figure 4. HIV-1 viral particle

In addition to structural proteins, HIV-1 particle carries an array of accessory proteins that are important for viral infectivity due to their antagonism of cellular restriction factors (RFs) (Figure 5) and interaction with cellular processes that are necessary for HIV infection. Some of these proteins are carried inside the cell by the viral particle, while others are synthesized de-novo upon viral transcription and translation. These proteins include: Vif, Vpr, Vpu, Nef, Tat and Rev. All of these proteins have multiple functions, which are described throughout

the text. Briefly, however, the most known functions of some of these proteins are outlined here. For example, Nef boosts the activity of transcription factor (TF) NF- $\kappa$ B early during infection to induce viral promoter transcription (see below) and downregulates CD3, CD4, CD8, CD28 and MHC-I expression to suppress adaptive immunity (Sauter and Kirchhoff, 2018). Vpu, on the other hand, inhibits NF- $\kappa$ B during later phases of infection to prevent transcription of anti-viral factors (Sauter and Kirchhoff, 2018). Vpu has also been shown to suppress the expression of Rf tetherin and HLA-B, important for adaptive immunity. Vpr blocks the cell cycle of dividing cells in G2 phases thus ensuring maximal activity of LTR promoter. In non-dividing cell, Vpr ensures nuclear import of viral pre-integration complex (González, 2017). Vif counteracts host cell Rf APOBEC3 (Nakano et al., 2017). Tat activates viral transcription and regulates viral mRNA splicing (Jablonski et al., 2010) and Rev promotes splicing and nuclear export of nascent viral mRNA (Freed, 2015).



**Figure 5. Interaction of HIV-1 accessory proteins with cellular factors. Adapted from (Sauter and Kirchhoff, 2018)**

## VIRAL LIFE CYCLE

HIV-1 life cycle begins with the entry of the viral particle mediated by the attachment to its receptor and co-receptors into the cell. After the attachment, the viral particle membrane fuses with the cellular plasma membrane to inject the viral capsid into the cytoplasm. Alternatively, HIV-1 has been reported to be able to enter the cell through fusion event in the endosome (de la Vega et al., 2011)(Fackler and Peterlin, 2000)(Carter et al., 2011). However, this remains controversial (Gregory B Melikyan, 2014). Once inside the cell, the viral capsid travels to the nuclear envelop while the genomic RNA is reverse transcribed into DNA. The viral DNA then integrates into the host chromosome. From here, the virus follows one of two fates: active transcription of viral gene products or latency. Latency is a state of transcriptional silencing of the virus. Transcription and subsequent translation of viral mRNAs forms structural and non-structural viral proteins followed by their trafficking and assembly of the viral particle at the plasma membrane. The viral particle buds at the plasma membrane, matures and re-infects another cell to initiate a new cycle of infection (Figure 6).

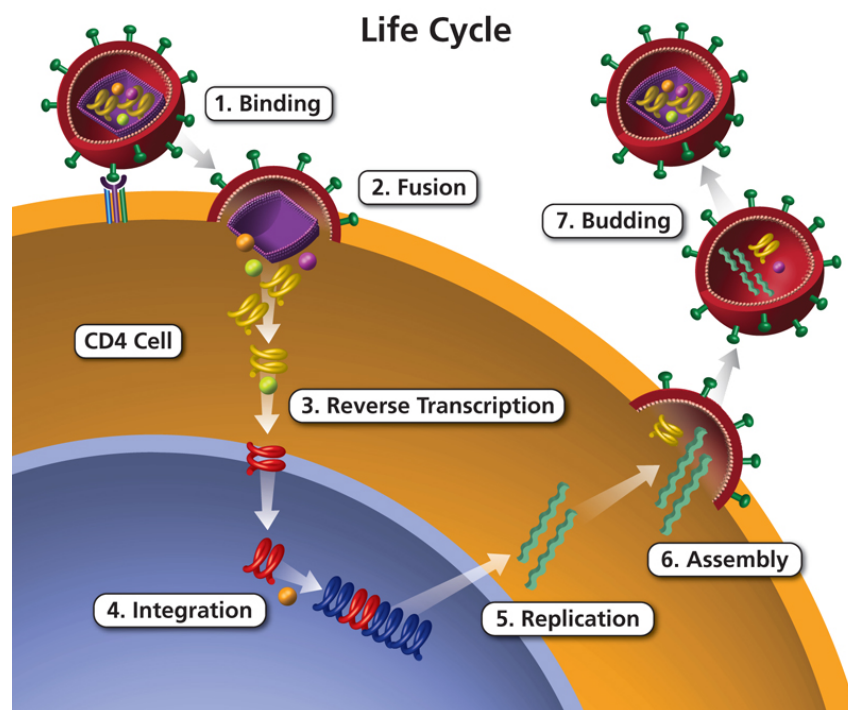


Figure 6. HIV-1 life cycle. Adapted from AIDSinfo.nih.gov

## ***Entry***

The entry of the HIV-1 viral particle into the cell is mediated via the attachment of the Env glycoprotein to its receptor CD4 and co-receptor CCR5 or CXCR4. Env is a glycosylated trimer of non-covalently attached gp120 and gp41 heterodimers. Env interacts with CD4 and its co-receptor via gp120. Viruses that use CCR5 co-receptor are called R5 viruses and viruses that use CXCR4 co-receptor are called X4 viruses. R5X4 viruses also exist.

Co-receptor binding on the cell surface exposes gp41 fusion peptide, which inserts itself into the cell membrane and, via the structural rearrangement, brings viral and cell membranes in close proximity resulting in the fusion of the viral capsid into the cytoplasm (Wilén et al., 2012).

## ***Reverse Transcription***

After viral core fusion into the cytoplasm, reverse transcription is initiated. Viral capsid surrounds genomic RNA and RT and protects viral genome from the recognition by the innate pattern recognition sensors in the cytoplasm. However, it is permeable to small molecules such as dNTPs, which are delivered inside the capsid to serve as reverse transcription substrates (Sleiman et al., 2012). Viral RT has a high affinity for dNTPs (Skasko et al., 2005), permitting reverse transcription to take place even in the limiting concentrations of dNTPs, which is the case for non-replicating cells such as macrophages (Diamond et al., 2004, p. 200).

Reverse transcription starts with the binding of tRNA<sup>Lys<sub>3</sub></sup> primer (carried in the viral particle from the previous host cell) to the primer binding site located close to the 5'-end of the genomic RNA (Sleiman et al., 2012). The DNA minus strand is synthesized by the RT using genomic RNA as a template. Nascent RNA-DNA duplex is recognized by the RT's RNaseH domain and RNA is cleaved at numerous sites. Only short RNA fragments (polypurine tracts) remain hybridized to the nascent DNA strand. These fragments serve as primers for the synthesis of the DNA plus strand and get subsequently degraded by the RNaseH activity of the RT (Amie et al., 2013).

## ***Integration***

Lentiviruses such as HIV-1 can infect both dividing and terminally differentiated non-dividing cells due to their ability to traverse the nuclear envelope (Fassati, 2006). Viral core enclosed by the CA travels through the cytoplasm to the nuclear pore intact, uncoated, or partially uncoating in the process (a matter of debate) while reverse transcription is taking place (Yamashita and Engelman, 2017)(Hilditch and Towers, 2014). The viral core docks at the nuclear pore via its interaction with the CA (Lusic and Siliciano, 2017). Pre-integration complex (PIC), which consists of viral DNA in complex with an IN multimer (an intrasome), CA, MA, Virus protein R (Vpr) and cellular proteins, traverses the nuclear pore through the nuclear pore complex (PNC) to enter the nucleus periphery. Vpr is a key regulator of viral nuclear import and is indispensable for the infection of non-dividing cells such as macrophages (Hattori et al., 1990)(Popov et al., 1998).

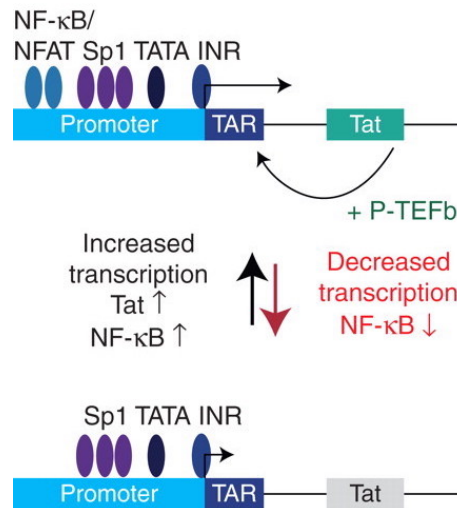
After the nuclear import the intrasome binds to the target cellular DNA where IN mediates viral DNA integration. IN cleaves 2 nucleotides from each 3' end of the DNA strand, which exposes chemically reactive -OH group, thereby creating a double strand break. IN then mediates strand transfer, a process by which viral DNA is recombined with the cellular DNA. The cellular machinery that normally repairs chromosomal DNA damage repairs single-stranded gaps that remain after the recombination event. The integration generally favors gene-dense, active transcription sites at the nuclear periphery (Marini et al., 2015) and was correlated with the level of splicing inside those sites (Singh et al., 2015) (Lusic and Siliciano, 2017).

## ***Transcription***

After integration, HIV-1 genome can either enter into the state of latency or be transcribed under the control of the 5' long terminal repeat (LTR) promoter. LTR promoter is a promiscuous promoter that mediates gene transcription under the control of cellular transcription factors (SP1, NFκB, NFAT and TBP are the most recognized, although others are known) (Pereira et al., 2000). LTR contains binding sites for these promoters as well as

additional functional domains such as the TATA box and the transactivation response element (TAR) element (Figure 7). Sp1 binding site and the TATA box are essential for transcription initiation and basal promoter activity (Ne et al., 2018). Binding of these transcription factors promotes the recruitment of histone-modifying enzymes that allow for transcriptionally permissive chromatin environment. This, in turn, facilitates the installation of transcriptional machinery on the LTR promoter (Ne et al., 2018).

Upon the first rounds of transcription from the HIV-1 LTR, low levels of the Transcriptional activator (Tat) is produced. Tat binds to the TAR element, a short transcript with the hairpin structure (Asamitsu et al., 2018). Tat initiates high levels of viral transcription via recruitment of Cdk9, which phosphorylates and activates RNA Polymerase II (Salerno, 2007) (Asamitsu et al., 2018). Tat also mediates transcriptional elongation and stabilizes viral mRNA (Asamitsu et al., 2018). Viral genome is first transcribed as a full-length precursor mRNA molecule, which can then undergo extensive alternative splicing to generate mature mRNAs for structural and non-structural proteins of the virus or serve as a genomic RNA molecule (Sertznig et al., 2018).



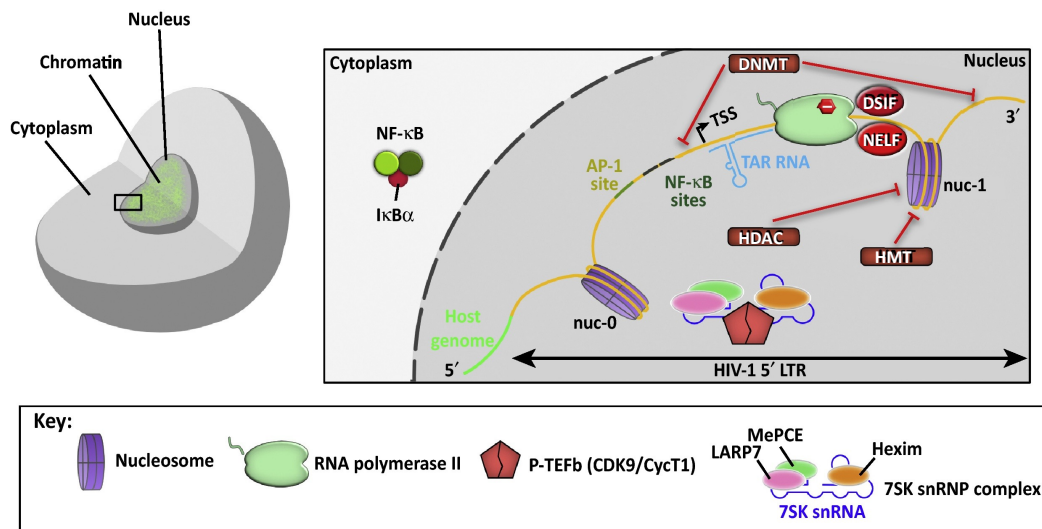
**Figure 7. 5' LTR promoter domains. Low levels of Tat are produced due to low level transcription from the LTR promoter. Tat binds to nascent TAR RNA transcript and recruits p-TEFb and other elongation factors to the transcription complex thus boosting transcription from the LTR promoter. The presence of the NF-κB, TATA, Sp1, NFAT and other transcription factors promotes HIV-1 transcription and their relative absence drives HIV-1 into latency by inhibiting transcription. Adapted from (Karn and Stoltzfus, 2012)**



## ***Latency***

After integration, HIV-1 can enter the state of latency through multiple mechanisms. These mechanisms inhibit the production of Tat, which normally stimulates transcription from viral LTR promoter. In general, the state of latency strongly depends on the transcriptional activity of the host gene containing the integration site. However, even in actively transcribed genes, several molecular mechanisms can inhibit viral transcription. These mechanisms act to remove TF or inhibit the activation of LTR promoter. For example, steric hindrance occurs when HIV-1 integrates downstream and in the same orientation as the host gene. In that case, RNA polymerase transcribing the host gene displaces the key TFs from the viral promoter that are necessary for transcription initiation (Darcis et al., 2017a).

Epigenetic mechanisms also play a key role in latency establishment (Figure 8). During latency, the integrated provirus exhibits inhibitory heterochromatin marks thus silencing active transcription. In particular, during latency, two nucleosomes (nuc-0 and nuc-1) are situated at the viral promoter irrespective of its integration site (Darcis et al., 2017a). Histone acetyl transferases (HATs) and histone deacetylases (HDACs) can acetylate or deacetylate respectively lysine residues in histone tails thus acting to remodel the chromatin and making the viral promoter more or less respectively accessible to transcription. Similarly, histone methyltransferases can induce transcriptional repression (H3K9 and H3K27 trimethylation) thus contributing to latency induction (Friedman et al., 2011; Imai et al., 2010). In addition to histone modifications, DNA methylation contributes to latency. During latency, HIV-1 promoter is hypermethylated at two CpG islands, which directly blocks access of TFs to the promoter, or induces repressive heterochromatin environment (Darcis et al., 2017a).



Trends in Immunology

**Figure 8. Mechanisms of HIV-1 latency induction. HIV-1 integrates in the periphery of the nucleus. During latency, transcription elongation by RNA polymerase II is blocked by nucleosome nuc-1 present after transcription start site (TSS). Repressive epigenetic environment consisting of DNA methylation (mediated by DNMT – DNA methyltransferase), histone deacetylation (mediated by HDACs – histone deacetylases) and methylation (mediated by HMT – histone methyltransferase) as well as sequestration of NF-κB in the cytoplasm and p-TEFb in an inactive complex induces viral latency. Other molecular factors such as DSIF - 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) sensitivity-inducing factor and NELF – negative elongation factor also negatively regulate HIV-1 transcription. Adapted from (Darcis et al., 2017b)**

Activation status of the infected cell also plays a critical role in latency establishment. Resting cells provide low supply of TFs and other molecules needed for efficient transcription. For example, cyclin T1 (a subunit of P-TEFb, HIV dependency factor important for viral transcription) is found in extremely low quantities in resting CD4+ T cells and the rest of the P-TEFb is sequestered to prevent its activity. Similarly, NF-κB is sequestered in the cytoplasm of unstimulated cells and is thus unavailable for transcription activation (Darcis et al., 2017a).

## ***Viral particle assembly, budding and maturation***

Once the viral mRNA is synthesized, it is transported from the nucleus to the cytoplasm where the viral proteins are translated. Gag polyprotein is synthesized as a precursor that is cleaved inside the viral particle by the protease (PR) into MA, CA, NC, p6 peptides as well as other small peptides (Freed, 2015). MA domain of the Gag polyprotein targets it to the plasma membrane, where it promotes incorporation of Env into the budding virion (Ono et al., 2004). CA domain drives Gag multimerization during particle assembly. NC contains two zinc finger-like domains that mediate its interaction with the viral genomic RNA. Unspliced viral RNA that serves as the genome is exported from the nucleus via its interaction with the Rev protein (Freed, 2015). NC acts as a nucleic acid chaperone and mediates RNA recruitment to the plasma membrane and its incorporation into the viral particle. Lastly, P6 peptide of the Gag polyprotein ensures Vpr incorporation and recruits endosomal sorting complex required for transport (ESCRT) which drives membrane fission during particle budding (Freed, 2015).

Env glycoprotein is also synthesized as a polyprotein precursor (gp160) that contains gp120 and gp41 proteins. Gp160 monomer trimerizes in the ER and is cleaved into gp120 and gp41 in the Golgi, after which it traffics through the secretory pathway to the plasma membrane (Checkley et al., 2011). Following cleavage, gp120 and gp41 remain associated by non-covalent interaction. In part to prevent the premature interaction of Env with its receptor CD4, viral Vpu protein binds to CD4 and mediates its proteosomal degradation. At the plasma membrane, gp120/gp41 are continuously recycled via the endosomal recycling pathway, which ensures low recognition by the immune system and low incorporation into the viral particle (Brandenberg et al., 2015).

GagPol is another precursor polyprotein that is synthesized and contains PR, RT and IN. The budded viral particle is immature and non-infectious with spherical capsid composed of Gag polyproteins. After budding, the viral particle matures, via the cleavage of Gag and GagPol polyproteins by the viral protease (PR) (Briggs et al., 2003). Following the cleavage of Gag by

PR, CA protein reassembles to form a conical shaped core around the viral genome and associated NC, RT and IN (Yamashita and Engelman, 2017).

## **CELLULAR DETERMINANTS OF HIV-1 INFECTION**

The ability of the HIV-1 to establish infection depends on the tight balance of intracellular restriction vs viral dependency factors that allow for efficient viral replication. Although several of these factors are known, this area remains under intense investigation today.

I start my discussion of HIV restriction factors with interferons, a family of immunomodulatory cytokines, which induce many known HIV restriction factors.

### ***INTERFERON***

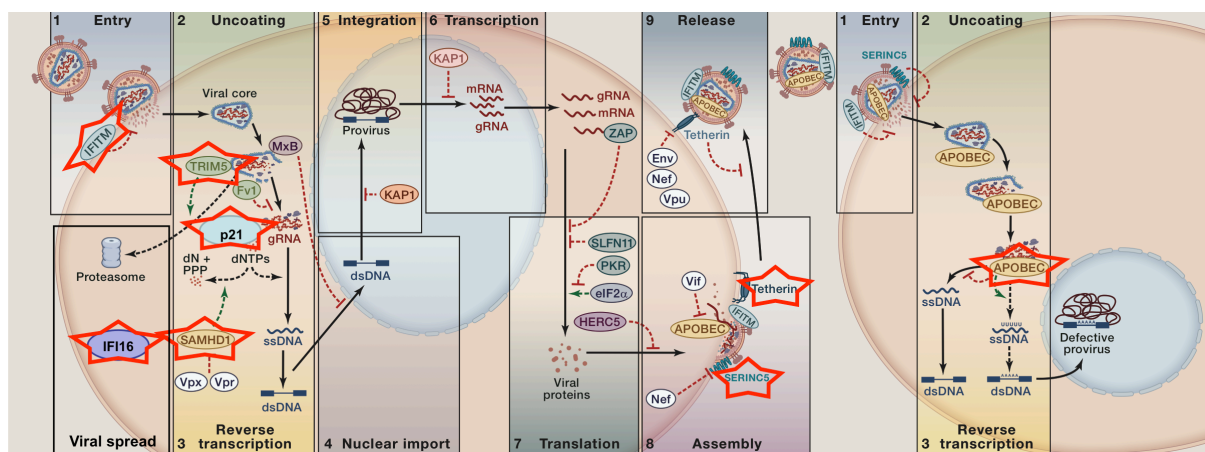
Interferons (IFNs) (type I, II and III) are a family of cytokines that are induced during viral infection. Type I IFNs (IFN-I) (IFN $\alpha$ , IFN $\beta$ , IFN $\omega$ , IFN $\epsilon$  and IFN $\kappa$ ) are most known for their anti-viral function. Upon binding to their cell surface receptors type I IFNs induce, among other functions, an anti-viral state through the induction of multiple interferon stimulated genes (ISGs) (Goodbourn et al., 2000). Some of these genes serve as restriction factors for HIV-1 infection (see below) (Doyle et al., 2015) (Goodbourn et al., 2000).

Upon HIV-1 infection, various pattern recognition molecules [ex. Interferon gamma inducible protein 16 (IFI16) and cyclic GMP-AMP synthase (cGAS) in the cytoplasm and toll-like receptor 7 (TLR7) in the endosome] recognize viral nucleic acid material and stimulate IFN-I production (Doyle et al., 2015). As a result, high levels of IFN- $\alpha$  is seen in the plasma of HIV-1 infected individuals during acute infection (Stacey et al., 2009), which has been postulated to be important in controlling initial phase of infection (Harris et al., 2010)(Sandler et al., 2014)(Soper et al., 2018). During chronic infection however, sustained IFN levels were shown to be associated with disease progression by driving chronic inflammation, immune activation and CD4+ T cell depletion (Jacquelin et al., 2009)(Harris et al., 2010)(Fraietta et al., 2013) (Soper et al., 2018). Animal and human trials of IFN treatment and IFN receptor block showed results consistent with the known role of IFN during the course of the disease. Treatment of macaques with IFN- $\alpha$ 2a before and during acute infection transiently prevented systemic infection but exacerbated infection during chronic treatment (Sandler et al., 2014).

Administration of IFN- $\alpha$ 2 to infected people resulted in transient decrease in plasma viremia but no long term benefit and even further decrease in CD4+ T cell counts (Utay and Douek, 2016).

## **HIV-1 RESTRICTION FACTORS**

Several HIV-1 restriction factors are known today. However, most of them are ineffective against the virus due to their efficient neutralization by viral factors (Figure 9).



**Figure 9. HIV-1 restriction factors and their known role in the viral life cycle. The restriction factors mentioned in the text are marked with red stars. SERINC and IFITM proteins prevent viral particle fusion; SAMHD1, p21 and APOBEC proteins inhibit reverse transcription; Tetherin inhibits budding and IFI16 prevents viral spread. Adapted from (Kluge et al., 2015)**

### **Entry**

**Serine Incorporator (SERINC)** proteins incorporate serine into cell membranes and facilitate synthesis of phosphatidylserine and sphingolipids (Ghimire et al., 2018). Of these, SERNIC3 and 5 were shown to restrict HIV-1 but are counteracted by viral protein Nef (Rosa et al., 2015)(Usami et al., 2015). In the absence of Nef, SERINC3/5 incorporate into the viral particle

plasma membrane and prevent their fusion (Rosa et al., 2015)(Usami et al., 2015)(Sood et al., 2017). Nef, by contrast, targets SERINC into late endosomal compartment and thus inhibits its incorporation into budding virions (Rosa et al., 2015).

**Interferon-induced transmembrane (IFITM) proteins** are a family of transmembrane proteins that have broad antiviral activity against many enveloped viruses (Shi et al., 2017). In HIV-1, IFITMs were shown to be effective against free virus infection when expressed on target cells (Lu et al., 2011) and against cell-associated viral infection when expressed on donor cells (Compton et al., 2014)(Tartour et al., 2014). They were also shown to be incorporated into viral particles (Compton et al., 2014) (Tartour et al., 2014). IFITM1-3 proteins restrict viral entry by poorly understood mechanisms, which may include alteration of membrane biophysical properties, cholesterol content (Shi et al., 2017) and inhibition of hemifusion states (Yu et al., 2015).

### ***Reverse transcription and integration***

***Sterile alpha motif and histidine-asparatate domain containing protein 1 (SAMHD1)*** has several known functions. Its triphosphohydrolase (dNTPase) enzymatic activity converts deoxynucleotide triphosphates (dNTPs) into deoxynucleosides (Powell et al., 2011), thus depleting dNTP substrates for viral RT (Lahouassa et al., 2012)(Goldstone et al., 2011). It also restricts HIV-1 infection via its 3'-5'-exoribonuclease (RNase) activity thus degrading HIV-1 genomic RNA (Ryoo et al., 2014)(Choi et al., 2015). The relative importance of these two functions for HIV-1 restriction is debated (Ryoo et al., 2014).

SAMHD1 is critical for HIV-1 restriction in myeloid cells (Hrecka et al., 2011)(Laguette et al., 2011) and in resting CD4+ T cells (Baldauf et al., 2012), where levels of active SAMHD1 are abundant. SAMHD1 is, however, ineffective at restricting HIV-1 in activated, cycling CD4+ T cells and cycling macrophages due to its lower levels and cell cycle dependent inhibitory phosphorylation at Thr592 (Ryoo et al., 2014)(Mlcochova et al., 2018) (Lindgren et al., 2018). It is also ineffective in SIV and HIV-2, where viral protein Vpx targets it for proteosomal degradation (Hrecka et al., 2011)(Laguette et al., 2011). Interestingly, induction of DNA

double strand breaks (DSB) was shown to decrease the levels of phosphorylated SAMHD1 and enhance its anti-viral function (Jáuregui and Landau, 2018) (Mlcochova et al., 2018).

**P21** is a cell cycle inhibitor, which, together with p53, is required to sustain G2 cycle cycle arrest after DNA damage through its inhibitory interaction with cyclin-dependent kinase 2 (CDK2) (Asada et al., 1999) (Bunz et al., 1998). Intriguingly, viral protein Vpr was reported to mediate cell cycle arrest in G2 phase via the induction of p21 expression, presumably to enhance LTR-driven transcription (Chowdhury et al., 2003). On the other hand, however, p21 inhibits CDK2 dependent phosphorylation of viral reverse transcriptase, which is necessary for efficient viral reverse transcription (Leng et al., 2014). P21, therefore, could have a complex role in HIV-1 infection depending on the viral infection stage and the host cell type.

In intravenous Ig (IVIG)-stimulated macrophages and mature DCs, p21 has been found, similarly to SAMHD1, to inhibit reverse transcription of HIV-1 (Bergamaschi et al., 2009) (Allouch et al., 2013) (Valle-Casuso et al., 2017). In contrast to SAMHD1, p21 does not directly hydrolyse dNTPs but rather inhibits their biosynthetic pathway via inhibition of dNTP biosynthetic enzymes RNR2, TYMs and TK1 (Allouch et al., 2013) (Valle-Casuso et al., 2017, p.). P21 has also been shown to increase phosphorylation of SAMHD1 (Allouch et al., 2014; Pauls et al., 2014) (Badia et al., 2016)(Valle-Casuso et al., 2017) in macrophages and to be induced in response to DNA damage (Mlcochova et al., 2018).

The role of p21 in HIV-1 infection of CD4+ T cells, however, is less clear. p21 mRNA was found to be upregulated in productively infected CD4+ T cells and was associated with reduction in p21-specific micro RNA (miR) expression (Guha et al., 2016). However, when p21 was induced by CD4+ T cell treatment with a cholesterol lowering drug (atorvastatin), this lead to p21-mediated inhibition of infection (Elahi et al., 2016). The p21 expression levels *in vivo* are also not always consistent with protection. The levels of p21 mRNA were shown to be higher in CD4+ T cells of HIV-1 infected patients who can spontaneously control infection (HIV controllers) in some reports (Sáez-Ciri3n et al., 2011)(Chen et al., 2011) but not in others (de



Pablo et al., 2015). Furthermore, even in controller cohorts where p21 expression was higher, it is unclear that p21 exerts a direct control of viral replication in these cells (Sáez-Ciri3n et al., 2011)(Chen et al., 2011).

***Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3 (APOBEC3) proteins*** belong to the family of single-stranded DNA deaminases (Simon et al., 2015, p. 20). Out of all known APOBEC3s, APOBEC3B, D, F, G and H are active against HIV-1, although APOBEC3G is the most potent (Holmes et al., 2007b). Despite their activity, HIV-1 has evolved an efficient way of counteracting APOBEC3 via its proteosomal degradation mediated by viral protein Vif thus drastically decreasing its incorporation into virions (Marin et al., 2003). In the absence of Vif, however, APOBEC3 proteins are packaged into the viral particles via the interaction with the genomic viral RNA and Gag (Zennou et al., 2004) where they mediate cytidine deamination of the viral genome (Mangeat et al., 2003)(Apolonia et al., 2015) (Sheehy et al., 2002). The edited viral genomes are aberrantly or not at all reverse transcribed during the next infectious cycle thus greatly reducing the amount of RT products (Mangeat et al., 2003).

Evidence exists, however, that antiviral action of APOBEC3 proteins may be partially independent of its deaminase activity and that it is able to inhibit RT directly (Bishop et al., 2008; Holmes et al., 2007b)(Bishop et al., 2006, p. 2006)(Holmes et al., 2007a). Various mechanisms by which this is accomplished have been proposed including interference with RT by steric hindrance (Bishop et al., 2008), inhibition of tRNA primer binding (Guo et al., 2006), defects in plus-strand DNA transfer (Mbisa et al., 2007) .

Even in the presence of Vif, some APOBEC3 manages to get incorporated in the viral particles, where it has been suggested to contribute to viral evolution (Holmes et al., 2007b). APOBEC3-created mutations have been shown to contribute to viral adaptation in natural infection by creating immune system escape mutations (Kim et al., 2014). To add further complexity, APOBEC3C and 3G have been shown to be upregulated in CD4+ T cells upon activation

(Refsland et al., 2010) and was recently found by us to correlate with HIV-1 infection in CD4+ T cells (Valle-Casuso, submitted; See Annex 2).

**TRIM5 $\alpha$**  binds to viral capsid upon entry accelerating its uncoating and thus exposing reverse transcription to the cellular environment of the cytoplasm (Malim and Bieniasz, 2012). This could have dual effect: it disrupts reverse transcription complex architecture and exposes viral genome to the recognition by PRRs thus blocking reverse transcription process. However, human TRIM5 $\alpha$  is only active against certain strains of mouse retrovirus but not HIV-1 or SIV (Malim and Bieniasz, 2012).

**Cyclic GMP-AMP synthase (cGAS)** is a cytosolic DNA recognition molecule (Altfeld and Gale Jr, 2015), which detects HIV and SIV DNA and triggers a signaling cascade that mediates interferon production (Gao et al., 2013). However, this PRR was not reported to directly restrict viral replication itself.

### ***Viral spread***

**Tetherin** also termed BST2 or CD317 is a transmembrane protein that has also GPI-membrane anchor (Kupzig et al., 2003). It is thus inserted into the cell plasma membrane and anchors onto the viral particle lipid bilayer therefore 'tethering' released viral particles onto the cell surface. Clustered viral particles are then re-endocytosed back into the cell (Neil et al., 2007). Tetherin is an IFN $\alpha$  inducible factor (Neil et al., 2006) that is effectively counteracted by viral Vpu of HIV-1 (Van Damme et al., 2008)(Neil et al., 2008) or Nef of SIV (Zhang et al., 2009)(Jia et al., 2009). The cytoplasmic tail of tetherin also induces NF $\kappa$ B proinflammatory signaling (Tokarev et al., 2013)(Galão et al., 2012). Thus, tetherin also acts as a viral sensor molecule.

**IFN-inducible protein 16 (IFI16)** is an interferon stimulated factor (ISG) whose localization is both cytosolic and nuclear (Altfeld and Gale Jr, 2015). IFI16 recognizes DNA products of HIV-1 RT and effectively recognizes HIV-1 DNA in macrophages, where it restricts HIV-1 replication

and stimulates production of interferons (Jakobsen et al., 2013). IFI16 also recognizes HIV-1 DNA in resting lymphoid tissue CD4+ T cells, where it mediates pyroptosis in abortively infected cells through the activation of caspase-1-dependent cell death pathway (Monroe et al., 2014). Additionally, IFI16 has now been characterized as direct transcriptional regulator of HIV-1 and other viruses (Jakobsen and Paludan, 2014). IFI16 has been reported to interact with Sp1 thus preventing its recruitment to promoters (Jakobsen and Paludan, 2014). In HIV-1, IFI16 is implicated in the reduction of transcription through interfering with LTR promoter activity and Gag processing (Telenti, 2014). This antiviral activity is counteracted by viral Vpr protein (Telenti, 2014).

## **HIV-1 DEPENDENCY FACTORS (HDFs)**

HDFs are intracellular factors that are important or essential for establishment of productive viral infection. Generally, HDFs are identified using genome-wide RNAi or CRISPR/Cas9 knockdown/knockout approaches. Although generating a lot of hits, such reports published by different groups rarely identify the same factors thus questioning the power of these approaches (Schott and König, 2017)(Pache et al., 2011)(Bushman et al., 2009). Moreover, most studies are carried out in cell lines, often of non-lymphoid origin thus limiting the extrapolation power to primary CD4<sup>+</sup> T cells and myeloid cells. However, despite these limitations, factors that have been identified by 2 or more reports yield more confidence (Bushman et al., 2009). Many other HDFs were identified serendipitously and have been since confirmed by others. Categorization of HDFs made it clear that HIV-1 relies heavily on several cellular pathways: cell activation, cell cycle progression, proliferation and metabolism. The role of cell survival pathways and apoptosis is also discussed.

### ***ENTRY AND TRAFFICKING***

In addition to entry through its receptor and co-receptors, HIV was shown to use other surface molecules to target cells. Env has been reported to bind to heparan sulfate proteoglycans on macrophages (Saphire et al., 2001). DCs were also shown to capture HIV via scavenger receptors such as DC-SIGN (Geijtenbeek et al., 2000), Siglec 1 (Izquierdo-Useros et al., 2014), Syndecan 3 (de Witte et al., 2007) and others.  $\alpha 4\beta 7$  integrin on peripheral T cells was also implicated in HIV entry (Arthos et al., 2008).

A whole cascade of actin polymerizing proteins gets activated upon the viral engagement of CD4 and the co-receptors. This leads to actin-dependent clustering of CD4 and CCR5/CXCR4 on the cell surface hence promoting viral entry (Ospina Stella and Turville, 2018). Fused viral capsid also depends on cellular cytoskeleton (actin, microtubules and associated proteins) for travelling through the cytoplasm towards the nucleus (Yamashita and Engelman, 2017).

## ***REVERSE TRANSCRIPTION***

CA protein that forms the viral capsid interacts with **cyclophilin A (CypA)** in the cytoplasm. CypA is a peptidyl prolyl isomerase, and has been reported to either enhance or inhibit viral infectivity depending on CA sequence and cell host type (Hilditch and Towers, 2014). For example, in DCs CypA has been shown to sense CA, promote DC maturation, launch type I IFN response and inhibit DC-mediated trans-infection of T cells (Manel et al., 2010). On the other hand, CypA was shown to be incorporated into viral particles via its interaction with Gag and to enhance viral infectivity (Thali et al., 1994)(Franke et al., 1994). In some cell types, it promotes capsid stability by delaying uncoating and promotes RT and integration (Yamashita and Engelman, 2017).

High level of dNTPs in cycling, activated cells allows the virus to establish infection more efficiently. For example, high levels of dNTPs in activated CD4+ T cells (2-5  $\mu$ M) allows for efficient HIV-1 reverse transcription upon entry (Diamond et al., 2004). On the other hand, dNTP levels are low in resting CD4+ T cells and myeloid cells (Triques and Stevenson, 2004) (Diamond et al., 2004), which reduces but not blocks efficient viral RT and replication.

## ***INTEGRATION***

Several cellular factors play an important role in import of PIC into the nucleus through the NPC. Some of the known factors include cyclophilin A, RANBP2, transportin 3 (TNPO3), nucleoporin 153 (NUP153) and nucleoporin 358 (NUP358) (Lusic and Siliciano, 2017). Barrier-to-integration (BAF) (Chen and Engelman, 1998) and High mobility group protein A1 (HMGA1) proteins have also been reported to be integration co-factors (Farnet and Bushman, 1997).

***Lens epithelium derived growth factor (LEDGF/p75)*** is probably the most known among a few specific integration HDFs described. This protein is a transcriptional activator although its precise function is not clear. It interacts with integrase of all lentiviruses (Poeschla, 2008),

targets it to the nucleus (Poeschla, 2008) and tethers it to the chromatin at active transcription unit sites (Meehan et al., 2009). Additionally, LEDGF/p75 was shown to interact with splicing factors and thus direct HIV-1 integration to transcription units that are highly spliced (Singh et al., 2015). Furthermore, it protects integrase from proteosomal degradation in the cytoplasm (Llano et al., 2004, p. 2).

## ***TRANSCRIPTION***

### ***Cellular transcription factors***

The presence of the activation stimuli ensures the supply of the right transcription factors at the LTR that allows initiation of viral transcription. The most known example of activation-dependent HIV-1 dependency factor is NFκB transcription factor. NF-κB is induced by cell activation (in T cells by TCR stimulation), among other stimuli, and induces transcription of many effector genes involved in the cell cycle, DNA repair, cell differentiation, anti-viral immunity (Heusinger and Kirchhoff, 2017). In the context of HIV-1, NF-κB is well known to bind to the viral LTR promoter where it initiates first lentiviral gene expression (Nabel and Baltimore, 1987) (Williams et al., 2007). On the other hand, it was shown that NF-κB can also promote HIV-1 latency (Williams et al., 2006). NF-κB can also initiate transcription of anti-viral gene effectors (Heusinger and Kirchhoff, 2017). Thus, HIV-1 modulates NFκB activity depending on the stage of the viral cycle and signaling context (Sauter and Kirchhoff, 2018). The activity of NFκB is boosted by viral Nef and Env during the early stages of infection to initiate viral transcription from the LTR promoter (Sauter et al., 2015), but is suppressed in a viral Vpu-dependent manner during the later stages to prevent transcription of anti-viral factors (Hotter et al., 2017; Sauter et al., 2015). Vpu prevents translocation of NFκB from the cytoplasm to the nucleus via stabilization of IκB, which is associated with the decrease in the interferon stimulated gene (ISG) transcription (Sauter et al., 2015).

## ***BUDDING***

Gag polyprotein interacts with PtdIns(4,5)P<sub>2</sub> during its recruitment to the plasma membrane and induces the recruitment of cholesterol and sphingolipids, which serve as the viral budding platforms (Freed, 2015). These lipids, therefore, serve as HDFs for viral budding.

The ESCRT machinery is indispensable for the fission of the budding viral particle from the cell surface. Gag has been found to interact with tumor susceptibility gene 101 (TSG101), part of the ESCRT-I machinery, and ALG2-interacting protein X (ALIX), part of the ESCRT-II and III (Freed, 2015).

## CONTROL OF HIV-1 WITH ANTI-RETROVIRAL THERAPY

Infection with HIV-1 in the absence of anti-retroviral therapy leads to the progression towards AIDS within 10 years of infection (“WHO | HIV/AIDS,” 2018). The rapid development of combination anti-retroviral therapy (cART) saved millions of lives around the globe. The treatment effectively lowers the blood viral load to undetectable levels (below 50 RNA copies/ml), drastically lowers chronic immune activation and inflammation associated with the disease. The life expectancy of virologically suppressed patients now approaches that of the general population (“Survival of HIV-positive patients starting antiretroviral therapy between 1996 and 2013,” 2017). It, hence, represents one of the major success stories of modern global health.

The first cases of AIDS were described in United States in the early 1980s. The first anti-retroviral medication, 3'-azido-3'-deoxythymidine (AZT) or zidovudine (ZDV) appeared on the market in 1987 (Lange and Ananworanich, 2014). AZT belongs to a nucleoside analog reverse transcriptase inhibitor (NRTI) drug class, which inhibit reverse transcriptase activity of the virus. Other NRTI compounds together with protease inhibitors (PIs) were developed in the subsequent years (Flexner, 1998). But It was not until 1996 that the triple combination therapy using 1 PI and 2 NRTIs was introduced that achieved durable long lasting control of the virus (Hammer et al., 1997)(Lange and Ananworanich, 2014). Today, there exist 23 antiretroviral drugs of various classes (Table 1) (including non-nucleoside reverse transcriptase inhibitors (NNRTIs), fusion inhibitors, integrase inhibitors and CCR5-blockers) that are used in cART (Palmisano and Vella, 2011), with the goal to reach durable suppression of the blood viral load below the standard limit of detection (50 copies/ml).

However, viral load rebound is observed in the blood upon cART interruption. This is due to reactivation of the replication competent virus found in persistently infected cells (see below). Moreover, detectable virus replication is found in gastrointestinal tract (Yukl et al., 2010) (Horiike et al., 2012) (Belmonte et al., 2007), lymph nodes (Horiike et al., 2012) (Chun et al., 2008), spleen (Horiike et al., 2012), genital tract and the central nervous system (Cory



et al., 2013). All of these compartments, hence, represent virus sanctuaries that harbor cells containing replication competent virus – *viral reservoirs*.

**Table 1. Anti-retroviral drugs approved by the US FDA between 1987-2014. Adopted from Eriksson et al, 2013**

Antiretroviral (abbreviation)	Drug class	Year of US FDA approval
Zidovudine (ZDV)	NRTI, nucleoside	1987
Didanosine (ddI)	NRTI, nucleoside	1991
Zalcitabine (ddC)	NRTI, nucleoside	1992
Stavudine (d4T)	NRTI, nucleoside	1994
Lamivudine (3TC)	NRTI, nucleoside	1995
Abacavir (ABC)	NRTI, nucleoside	1998
Tenofovir disoproxil fumarate (TDF)	NRTI, nucleotide	2001
Emtricitabine (FTC)	NRTI, nucleoside	2003
Saquinavir (SQV)	PI	1995
Ritonavir (RTV or r)	PI	1996
Indinavir (IDV)	PI	1996
Nelfinavir (NFV)	PI	1997
Amprenavir (APV)	PI	1999
Fosamprenavir (fos-APV)	PI	2003
Lopinavir (LVP)	PI	2000
Tipranavir (TPV)	PI	2005
Darunavir (DRV)	PI	2006
Nevirapine (NVP)	NNRTI	1996
Delavirdine (DLV)	NNRTI	1997
Efavirenz (EFV)	NNRTI	1998
Etravirine (ETV)	NNRTI	2008
Rilpivirine (RPV)	NNRTI	2011
Enfuvirtide (T20)	Fusion inhibitor	2003
Maraviroc (MVC)	CCR5-blocker	2007
Raltegravir (RAL)	Integrase inhibitor	2007
Elvitegravir (EVT)	Integrase inhibitor	2012
Dolutegravir (DTG)	Integrase inhibitor	2013

NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; US FDA, United States Food and Drug Administration.

## ***BARRIERS TO ELIMINATION OF HIV-1 RESERVOIRS***

### ***Early seeding of reservoirs***

The first reservoirs are seeded in mucosal tissues within hours of infection from where the virus rapidly spreads to the local draining lymph nodes (Hu et al., 2000). At these and other tissues sites, the reservoirs are seeded within hours-days of infection (Stieh et al., 2016) (Haase, 2010)(Whitney et al., 2014). Because the reservoir seeding precedes clinical signs of disease, in most cases the initiation of therapy misses the critical window of opportunity to prevent reservoir establishment. However, early cART initiation has been shown to limit the size of the seeded reservoir that could lead to the spontaneous control of infection upon therapy interruption (Ananworanich et al., 2015)(Sáez-Cirión et al., 2013). The determinants of such control are currently being explored.

### ***Viral latency***

Indeed, cART does not cure HIV but only effectively prevents new rounds of infection from taking place. However, integrated, replication competent virus persists in latently infected cells (Dahabieh et al., 2015). Virus reactivation and new rounds of infection from this source happen during therapy interruption. CD4+ T cell activation due to antigen recognition and cytokine stimulation during cART may also play a role (Ruelas and Greene, 2013). The most accepted model is that latency is established after productive infection of activated CD4+ T cells that revert back to the resting state or of effector CD4+ T cells that become resting memory CD4+ T cells (Sengupta and Siliciano, 2018; Shan et al., 2017). However, an alternative model suggests that latency can be established directly in activated and resting CD4+ T cells (Chavez et al., 2015).

### ***Persistence of infected cell and ongoing re-seeding of reservoirs***

Long half-life of persistently infected cells is thought to be one of the major contributing factors to persistence of HIV-1 reservoirs. Half-life of infected resting memory CD4+ T cells on cART was proposed to be 3.7 years (Siliciano et al., 2003)(Crooks et al., 2015), although could be much longer in tissues. It is thus doubtful that even a life time of treatment would suffice

to eradicate this reservoir. Some CD4+ T cell subsets contribute more to the blood reservoir due to their long  $t_{1/2}$ : central memory CD4+ T (Cm) and Tscm cells with half life of months to years, while Tm and Tem subsets are more short lived (Mikhailova, 2018). Tm and Tem, however, were shown to maintain the reservoir by low level ongoing proliferation (Chomont et al., 2009a)(Hiener et al., 2017). Moreover, infected tissue macrophages and microglial cells could also greatly contribute to reservoir persistence due to their long half-life. Macrophages and microglial cells were reported to survive for months to years in different body compartments, although their  $t_{1/2}$  and infection status on cART is poorly known (Mikhailova, 2018).

### ***Tissue penetration of anti-retroviral drugs and residual viral replication***

Another barrier to clearance of viral reservoirs on cART is unequal and sometimes inefficient drug penetration into tissues. Multiple studies showed reduced penetration (2-50 times lower) of anti-retroviral drugs into CNS, lymph nodes and genital organs when compared to drug concentrations in the plasma (Solas et al., 2003) (Mascio et al., 2009)(Fletcher et al., 2014)(Sawchuk and Yang, 1999)(Gray et al., 2013)(Estes et al., 2017). CNS is especially affected (Sawchuk and Yang, 1999), hence representing a distinct compartment where the virus evolves separately and represents a genetically distinct reservoir (Dahl et al., 2014)(Gianella et al., 2016)(Edén et al., 2010)(Canestri et al., 2010)(Gama et al., 2017). Moreover, it is now known that cART is less efficient against cell-to-cell viral transmission as in the case of viral spread from dendritic cells to T cells (Kim et al., 2018).

Although debated, ongoing viral replication in tissue sanctuary sites relatively inaccessible by cART and immune system is also thought to contribute to reservoir persistence (Lorenzo-Redondo et al., 2016). Low levels of ongoing viral replication was observed in lymphoid tissue compartment despite effective suppression of blood viremia (Estes et al., 2017)(Lorenzo-Redondo et al., 2016). The virus has been shown to persist in female genital tract and is detected in genital secretions despite cART (Launay et al., 2011). Similarly, male reproductive

tract was identified as a viral reservoir where, similarly to the CNS, HIV-1 has been shown to evolve independently (Tortorec and Dejudcq-Rainsford, 2010), support ongoing viral replication despite therapy (Tortorec and Dejudcq-Rainsford, 2010) and to be intermittently shed in the semen (Svicher et al., 2014). In these tissues, resident macrophages and CD4+ T cells have been identified as viral carriers (Tortorec and Dejudcq-Rainsford, 2010).

## **WHICH CELL POPULATIONS CONSTITUTE THE HIV-1 RESERVOIR?**

### ***CD4+ T CELLS***

#### ***CD4+ T cell subset diversity***

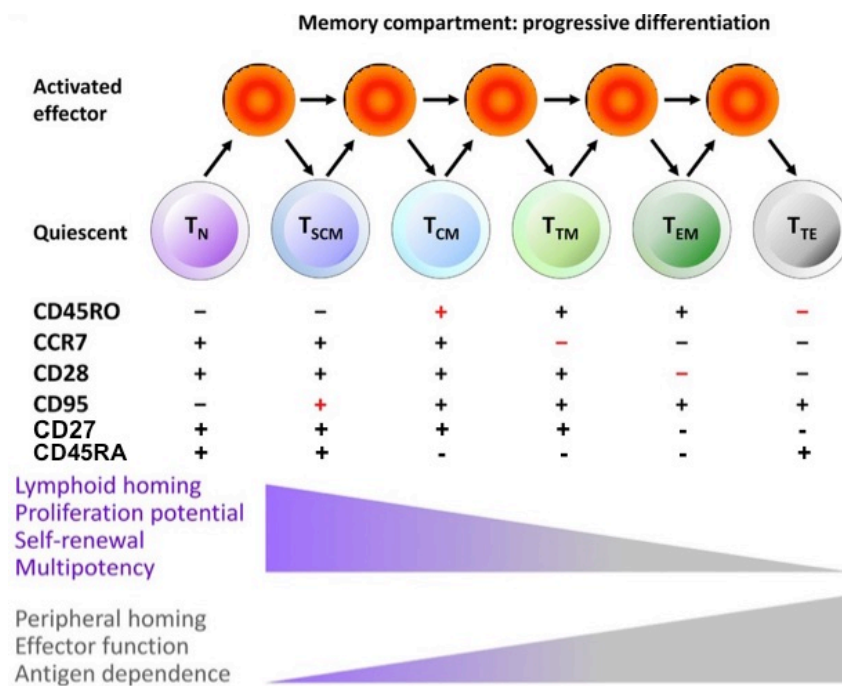
CD4+ T cells are major targets of HIV-1, where the virus replicates efficiently ultimately resulting in the depletion of these cells during the progression of infection. Despite the expression of CD4 and CCR5 – receptor and co-receptor of HIV – on their surface, different subsets of CD4+ T cells support productive infection at different rates. For example, resting and naive CD4+ T cells are highly restrictive to infection, while activated and memory subsets are permissive to infection. Here, I first outline the state of knowledge about the diversity of CD4+ T cells. I then present what is known about relative contribution of different CD4+ T cell subsets to HIV-1 reservoir.

Current model of T cell development indicates that after positive and negative selection in the thymus, CD4+ T cells enter blood as mature naïve cells (T<sub>n</sub>). Each of the cell clones contains a given T cell receptor (TCR) Ag specificity. T cells recirculate through secondary lymphoid organs where they are primed by Ag-bearing dendritic cells (DCs), differentiate and proliferate. Primed T cells then migrate to target tissues where they further proliferate and differentiate into different types of effector cells that mount an effective immune response (Mahnke et al., 2013)(Mueller et al., 2013). A combination of different activation signals (cytokines, co-stimulation molecules, TCR stimulation, etc) and the type of Ag dictates the subtype of effector cell and the tissue localization of the primed T cell. Several effector CD4+

T cell subtypes are known: Th1, Th2, Th17, Th22, Tfh, Tregs and cytotoxic CD4+ T cells (Gagliani and Huber, 2017; Vella et al., 2017). Briefly and simplistically, Th1 cells provide CD8+ T cell help; Th2 cells control parasitic infections and mediate tissue repair; Th17 control bacterial infections in the GI tract; Tfh cells mount B cell help in the lymphoid nodes; and Tregs downmodulate immune responses (Gagliani and Huber, 2017).

Following Ag clearance, more than 95% of effector cells die, leaving behind a pool of effector cells that will differentiate into resting memory cells. These cells retain a long-term Ag memory, meaning that they have high potential to quickly and effectively mount a secondary immune response upon Ag rechallenge (measured by proliferation, cytokine secretion, cytotoxicity and enhanced tissue migratory capacity) (Mahnke et al., 2013)(Mueller et al., 2013). Human T cell memory subsets follow a differentiation continuum from Tn cells through stem cell like memory (Tscm), central memory (Cm), transitional memory (Tm), effector memory (Em) to effector cells (Figure 10) (Mahnke et al., 2013). Tn, Tscm and Cm express lymph node homing receptors CD62L and CD27, which permits them to recirculate through the lymphoid tissues, be primed by DCs and initiate an adaptive immune response. Generally, memory T cell subsets are distinguished from their naïve counterparts by the expression of CD45RO and the lack of expression of CD45RA surface markers (Mahnke et al., 2013). CCR7 was further shown to divide Em from Cm cells. CCR7+ Cm cells have high proliferative capacity, lack immediate effector functions (rapid secretion of effector cytokines such as IFN $\gamma$ , IL-4 and IL-5) and secrete high levels of IL-2 (homeostatic proliferation cytokine) (Sallusto et al., 1999). Cm, together with Tscm cells, are thought to maintain long-lived T cell memory pool. CCR7- Em cells, on the contrary, express receptors required for homing to inflamed peripheral tissues, rapidly secrete effector cytokines in response to Ag challenge and secrete low levels of IL-2 (Sallusto et al., 1999). CD27 marker, in combination with CCR7 is used to distinguish a fourth memory population, Tm cells. These cells are CD27+ CCR7- and are thought to represent an intermediate stage of differentiation between Cm and Em subsets. In addition, tissue resident memory T (Trm) cells, initially defined by high expression of CD69 and CD103, are now known (Mueller and Mackay, 2016). These cells have a distinct transcriptional profile, and thus are different, from Cm and Em cells (although also derived from naïve precursors). They do not appear to recirculate through the blood and lymph, have

minimal turnover and high longevity and mount local tissue immune responses (Mueller et al., 2013)(Mueller and Mackay, 2016).



**Figure 10. CD4+ T cell subsets. Adapted from (Mahnke et al., 2013)**

### ***CD4+ T cell reservoir in blood***

Initiation of cART is accompanied by the rapid drop of blood viremia due to the clearance of free viral particle and productively infected cells (Perelson et al., 1997). However, the cell reservoir carrying virus remains. This reservoir is thought to be mostly composed of resting memory CD4+ T cells (Siliciano et al., 2003)(Finzi et al., 1997). Indeed, replication competent virus can be produced upon stimulation (with PHA or anti-CD3/anti-CD28 Ab) of resting CD4+ T cells from patients receiving long-term (years) cART (Chun et al., 1997)(Finzi et al., 1997)(Wong et al., 1997). This resting CD4+ T cell reservoir has been determined to consist mostly of central and transition memory CD4+ T cells with lesser contribution of effector memory CD4+ T cells (Chomont et al., 2009a). Recently, however, the importance of Em cell subset is beginning to be underscored. First, Em cells are best able to support productive viral infection *in vitro* (Valle-Casuso, submitted; see Annex 2). They are now thought to be the

source of latent Cm reservoir due to the differentiation of Em into Cm cells (Cheung et al., 2018; Shan et al., 2017). Moreover, they were recently found to contain highest proportion of full length viral genomes in virally suppressed patients (Hiener et al., 2017). HIV-specific memory CD4+ T cells were also shown to contain higher proportion of viral DNA than other subsets although replication competence of these viruses is unknown (Douek et al., 2002). Stem cell like memory CD4+ T cells (Tscm) may also have an important contribution to HIV-1 persistence due to their pluripotency potential and long half-life (Buzon et al., 2014).

### ***CD4+ T cell reservoir in the tissues***

Mucosal and gut CCR6+ Th17 cells are preferentially infected (Stieh et al., 2016), depleted first from the gut mucosa and are not fully recovered during cART (Brenchley et al., 2008). These cells were shown to have more favourable intracellular HIV-1 dependency factor (HDF) environment than other CD4+ T cell subsets (Cleret-Buhot et al., 2015, p.). During cART, lymphoid tissues, including lymph nodes and gut associated lymphoid structures, have been found to carry infected CD4+ T cells (Chun et al., 2008) (Perreau et al., 2013) (Banga et al., 2016). For example, T follicular helper (Tfh) in germinal centers are relatively inaccessible to cytotoxic CD8+ T cell activity and are thus deemed an important reservoir component (Perreau et al., 2013) (Banga et al., 2016). Tissue resident and other long-lived memory CD4+ T cell populations undoubtedly contribute to latent HIV-1 reservoir in lymphoid and non-lymphoid tissues but the precise identity of these cells remains to be characterized. In other tissues such as adipose, memory CD4+ T cells have also been shown to carry viral nucleic acid material (Couturier et al., 2015) and replication competent virus (Damouche et al., 2015). High proportion of infected Tfh cells have been detected in SIV infected macaques in cervical lymph nodes (Dave et al., 2018) indicating that CD4+ T cells infiltrating the CNS could be an important component reservoir (Kivisäkk et al., 2003).

## **BLOOD AND TISSUE MYELOID CELLS**

Genetic studies on viral diversity during viral rebound upon treatment interruption have demonstrated that resting CD4<sup>+</sup> T cells are not the only HIV-1 reservoir (Chun et al., 2000) (Dybul et al., 2003). Despite difficulty of infecting monocytes *in vitro* due to their low CD4 and CCR5 expression and resistance to viral replication, infected monocytes were found to persist during cART (Zhu et al., 2002) (Lambotte et al., 2000). These monocytes were shown to be capable of migration into tissues where they differentiate into macrophages, which provide more favorable intracellular viral replication environment (Palmisano and Vella, 2011).

Although they are able to be infected, macrophages express lower CD4, CCR5 and CXCR4 levels and provide more hostile intracellular environment to the virus than activated CD4<sup>+</sup> T cells (Jayakumar et al., 2005)(Lee et al., 1999). Tissue macrophages were shown to carry viral nucleic acids (Jambo et al., 2014) and persist in multiple tissues despite cART treatment (Araínga et al., 2017)(Matusali et al., 2015)(Cribbs et al., 2014)(Zalar et al., 2010). Importantly, macrophages from different tissue sources appear to have different susceptibility to productive infection. For example, alveolar (Jambo et al., 2014) and vaginal (Shen et al., 2009) macrophages are highly susceptible but not intestinal macrophages (Shen et al., 2011, 2009). Macrophages probably have an important contribution to HIV reservoirs due to their long  $t_{1/2}$ .

Dendritic cells are thought to be relatively resistant to productive infection (Manel et al., 2010), with mature DCs being more resistant than immature DCs (Valle-Casuso et al., 2017). In addition to being infected, DCs capture viral particles and transport them to the lymph nodes where they mediate CD4<sup>+</sup> T cell trans-infection (Kwon et al., 2002). Follicular dendritic cells were shown to capture and hold infectious virus for at least months even during cART (Fletcher et al., 2014) (Smith et al., 2001).



## ***CELLS OF THE CENTRAL NERVOUS SYSTEM***

CNS as a replication-competent HIV-1 reservoir has been disputed due to difficulties, until recently (Avalos et al., 2017), of isolating infectious virus from this compartment (Gray et al., 2014). However, several cell populations in the CNS have been clearly shown to be susceptible to infection and to carry proviruses.

Microglial cells are of embryonic macrophage origin and serve as phagocytic and antigen presenting cells of the CNS (Lannes et al., 2017). They express CD4, CXCR4 and CCR5 (He et al., 1997, p. 3)(He et al., 1997, p. 3)(Lavi et al., 1997) and are thus susceptible to infection. Infected microglial cells were detected *in vivo* in untreated people and monkeys (Churchill et al., 2006) (Cosenza; et al., 2002) (Llewellyn et al., 2018). Because they are extremely long-lived (years) (Gray et al., 2014) and are relatively isolated with regards to drug and immunity penetration, it is possible that these cells retain replication-competent virus on cART.

Perivascular macrophages, are derived from blood circulating monocytes. They have been described to be the primary targets of SIV infection during peak viremia in acute phase of infection (Williams et al., 2001). HIV-1 DNA has also been detected in perivascular macrophages of humans (Thompson et al., 2011). However, their moderate turnover of 2-3 months (Williams et al., 2001) could mean that they make a lesser contribution to reservoirs under cART than other populations.

Astrocytes positive for viral antigens and DNA were detected in infected brain tissue sections (Churchill et al., 2009) (Churchill et al., 2006)(K. A. Thompson et al., 2001) although they do not express CD4 or CCR5 (Russell et al., 2017) (Sabri et al., 1999). It has been recently shown, however, that astrocytes phagocytose viral particles and even infected cells and hence could be an important viral reservoir (Russell et al., 2017).

# IMMUNO-METABOLISM OF CD4+ T CELLS AND HIV-1 INFECTION

## GENERAL OVERVIEW OF IMMUNO-METABOLISM IN CD4+ T CELLS

Mounting an effective immune response requires drastic changes in CD4+ T cell activation, differentiation and proliferation. These processes are accompanied by changes in the cell's metabolic requirements. Thus, different stages of CD4+ T cell differentiation and activation are supported by distinct metabolic programs that allow the cell to support its function but also respond to local tissue environmental pressures (Dumitru et al., 2018).

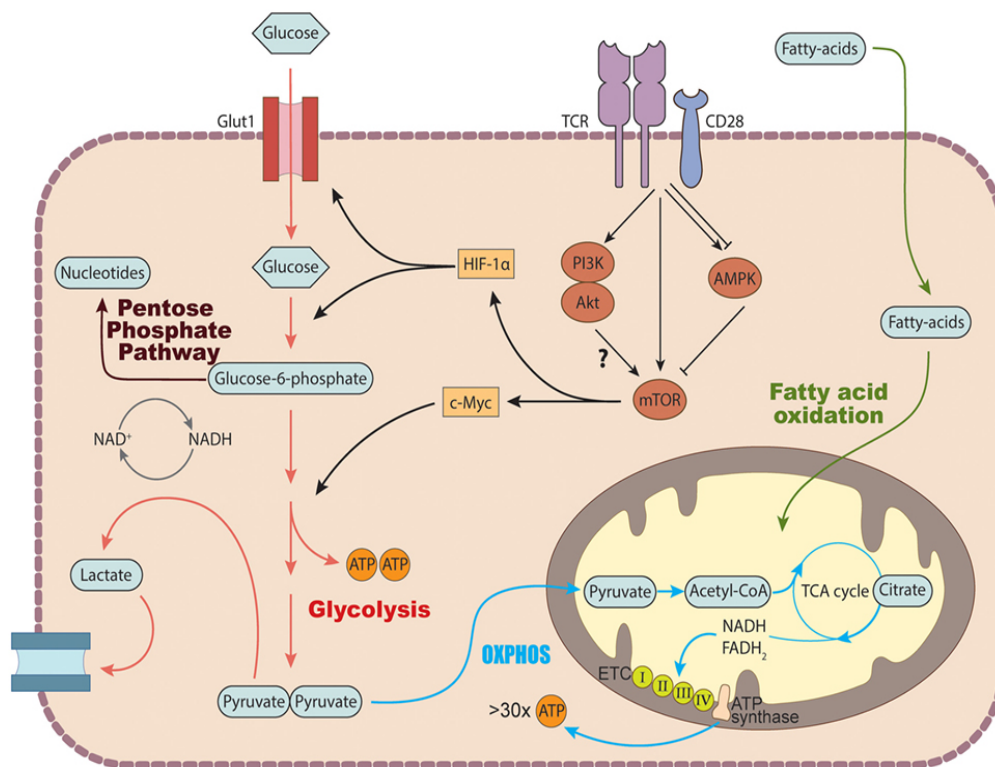
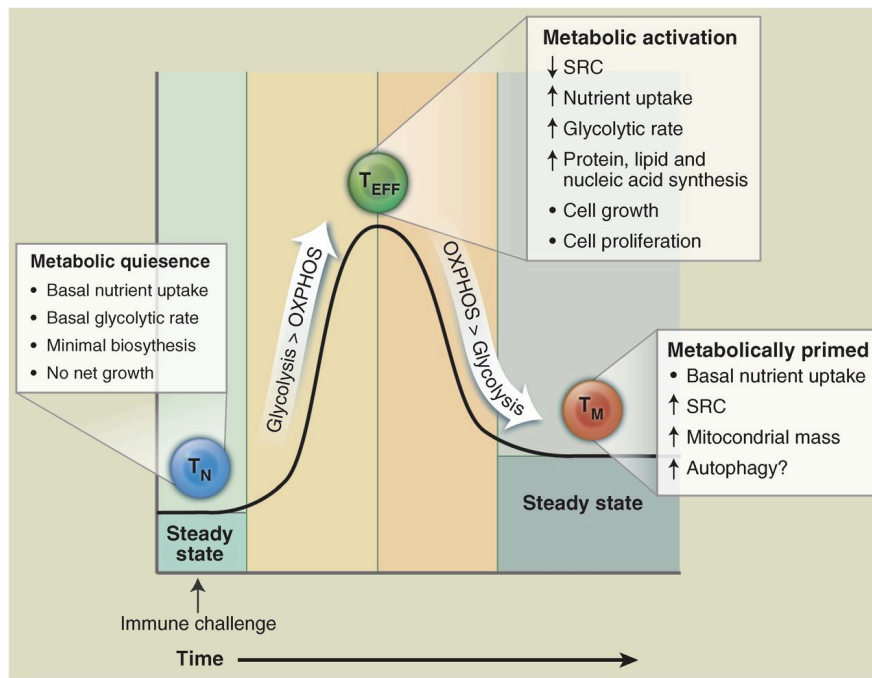


Figure 11. Metabolic pathways in CD4+ T cells. Adapted from (Dumitru et al., 2018)

Quiescent Tn cells rely predominantly on mitochondrial respiration to fuel energy production (Figure 11 and Figure 12) (Pearce et al., 2013). During this process, the available nutrients (glucose, fatty and amino acids) are broken down and their metabolic intermediates are fed into the tricarboxylic acid (TCA) cycle in the mitochondria. NADH and FADH<sub>2</sub> produced in the TCA cycle feed their electrons through the electron transport chain located in the inner mitochondrial membrane to drive the generation of the proton gradient across the

membrane, which fuels ATP production by ATP synthase (a process called oxidative phosphorylation (OXPHOS)). OXPHOS generates high number of ATP molecules (about 30 ATP per 1 glucose molecule) and is thus extremely energy efficient. While both OXPHOS and glycolysis are upregulated during T cell activation, relative contribution of OXPHOS to cellular metabolism is decreased and aerobic glycolysis becomes a dominant pathway (Figure 12). Glycolysis is less energetically efficient than OXPHOS (2 molecules of ATP per molecule of glucose) but provides biosynthetic intermediates more readily. Glycolysis (together with TCA cycle), a breakdown pathway of glucose in the cytoplasm, generates biosynthetic intermediates that are funneled through different metabolic pathways such as pentose phosphate (PPP), serine biosynthesis, hexosamine and fatty acid synthesis pathways (Almeida et al., 2016). These pathways serve to eventually synthesize molecular building blocks of the cell such as nucleotides (PPP pathway), amino acids (serine biosynthesis pathway) and fatty acids. Lactate that is generated as a final product of aerobic glycolysis, is exported from the cell.

Effector and regulatory T cells rely on different metabolic pathway for their function. For example, effector cells such as Th1, Th2 and Th17 utilize high rates of glycolysis while Tregs rely preferentially on fatty acid oxidation (FAO) pathways (Dumitru et al., 2018). As effector cells differentiate into memory cells, their metabolism reverts back from high reliance on glycolysis to OXPHOS and FAO (Figure 12).



**Figure 12. Changes in T cell metabolism according to differentiation stage. Adapted from (Pearce et al., 2013)**

## ***THE ROLE OF CD4+ T CELL METABOLISM IN HIV-1 INFECTION***

In parallel to the activation and cell cycle status, the cell's metabolic state plays a key role in efficient HIV-1 replication. Upregulation of Glut1 expression on CD4+ T cell surface has been directly linked to susceptibility of the cells to HIV-1 infection (Loisel-Meyer et al., 2012a). Moreover, Glut1 levels have been shown to be increased on CD4+ T cells during chronic HIV-1 infection and not completely return to baseline upon treatment (Palmer et al., 2012). Dependence of HIV-1 infection on Glut1 expression is thought to be linked to its role as a glucose transporter. Consequentially, successful HIV-1 replication has been shown to rely on glycolysis (Hegedus et al., 2014a)(Hollenbaugh et al., 2011a)(Valle-Casuso, submitted; see Annex 2), which has been shown to supply metabolic intermediates for the viral cycle (Valle-Casuso, submitted; see Annex 2). More recently, activation of PI3K metabolic pathway, and

its surrogate marker OX40 were proposed to be a better predictor to viral infection susceptibility in combination with Glut1 expression than Glut1 expression alone (Palmer et al., 2017).

As mentioned before, activated CD4<sup>+</sup> T cells rely heavily on glycolysis for their function. TCR stimulation alone is sufficient to upregulate Glut1 glucose transporter as well as other glutamine and amino acid transporters on the cell surface (Almeida et al., 2016)(Pearce et al., 2013). Moreover, CD28 co-stimulation was directly linked to upregulation of glycolytic pathway (Jacobs et al., 2008)(Frauwirth et al., 2002). T cell activation also leads to the induction of mammalian target of rapamycin (mTOR) pathways. mTOR is a serine/threonine kinase that can exist as part of two protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), both of which integrate different environmental and T cell activation signals to mount an appropriate metabolic and differentiation responses (Jones and Pearce, 2017). mTORC1, for example, induces the expression of HIF-1 $\alpha$  transcription factor, which upregulates glycolytic pathways and glucose transporters. mTORC1 has been shown to be important for Th1 differentiation, while mTORC2 seems to be important for Th2 differentiation and survival (Almeida et al., 2016). Other anabolic pathways such as glutaminolysis are upregulated upon T cell activation as well.

A central regulator of metabolic processes, mTOR was shown to have potential for therapeutic intervention during HIV-1 infection. mTORC1 inhibitor rapamycin was shown to potentiate anti-viral effect of CCR5 antagonist vicriviroc (Heredia et al., 2008) and to inhibit viral particle production and viral transcription (Roy et al., 2002)(Rai et al., 2013). INK128 compound, which blocks the activity of both mTORC1 and mTORC2 was shown to inhibit entry, reverse transcription and integration of R5 but not X4 viruses and be effective at lowering viral load by several logs in a humanized mouse model (Heredia et al., 2015). Moreover, viral infection was identified to activate mTOR in a Tat dependent manner (Kumar et al., 2016). Additionally, mTOR was demonstrated to control HIV-1 latency through phosphorylation of p-TEFb subunit CDK9. Inhibition of mTOR by Torin1 and pp242 inhibited

latent HIV-1 reactivation and prevented Tat-dependent and Tat-independent HIV-1 transcription (Besnard et al., 2016).

In light of this knowledge, and the failure of “shock and kill” approaches to clear viral reservoirs, “block and lock” model now proposes to limit the reactivation of latent HIV-1 reservoir at least partially through inhibition of mTOR activity (Darcis et al., 2017b; Palmer et al., 2018). Alternatively, the “starve” model proposes limiting the metabolic activity of reservoir cells to limit their homeostatic proliferation (Palmer et al., 2018; Palmer and Crowe, 2016).

# **THE ROLE OF CELL SURVIVAL VS CELL DEATH IN HIV-1 INFECTION**

Cell death clearly plays an important role in the pathogenesis of HIV-1 infection. Drastic depletion of CD4+ T cells is well documented both during acute and chronic stages of infection. However, precise molecular reasons for the depletion of CD4+ T cells during HIV-1 pathology are still poorly understood. Moreover, despite high levels of mortality in both infected and non-infected 'bystander' cell populations, the virus persists in surviving cells. Therefore, how some cells succumb to virus induced cytotoxicity while others survive remains an important open question in the field. Below, I present some of the general cell death pathways that are most relevant in the context of HIV-1 infection, and describe the current stage of knowledge of the role of the viral components in molecular pathways of cell death.

## ***GENERAL OVERVIEW OF CELL DEATH MECHANISMS***

Twelve forms of regulated cell death are currently recognized by the Nomenclature Committee on Cell Death (Galluzzi et al., 2018). Morphologically, cell death is classified into three forms. 1) Apoptosis exhibits characteristic cell shrinkage, chromatin condensation, nuclear fragmentation, plasma membrane blebbing and the formation of apoptotic bodies (small vesicles). 2) Autophagy is characterized by extensive cytoplasmic vacuolization. 3) Necroptosis involves swelling of the mitochondria and rupture of the plasma membrane. However, molecular pathways governing cell death processes are highly diverse and go beyond morphological characterization (Galluzzi et al., 2018). Here I review only three cell death pathways: apoptosis, pyroptosis and necroptosis - pathways most recognized in the context of HIV-1.

### ***Apoptosis***

Intrinsic apoptosis is initiated by various environmental perturbations such as growth factor withdrawal, DNA damage, endoplasmic reticulum stress, etc. BH3-only proteins are initiators of apoptotic pathway (Figure 13). Some of the examples of this family include BCL2 associated death promoter (BAD), BCL2 interacting mediator of cell death (BIM), p53-upregulated

mediator of apoptosis (PUMA), NOXA and truncated BH3-interacting domain death (tBID). They are induced either transcriptionally or post-translationally by cytotoxic stress signals. They either neutralize the pro-survival BCL2 family proteins (ex. MCL1, BCL2, BCL-W, etc) or directly activate apoptotic initiators BCL2 associated X protein (BAX) and BCL2 antagonist/killer (BAK) (Czabotar et al., 2014).

In healthy cells, BAK is a transmembrane protein residing in the outer mitochondrial membrane (OMM) and BAX shuttles between OMM and the cytoplasm. During activation of apoptotic program, BAX and BAK change their conformation to form homo-oligomers that form pores on the OMM, which mediates irreversible mitochondrial outer membrane permeabilization (MOMP). MOMP releases mitochondrial apoptogenic factors like cytochrome c and apoptosis-inducing factor (AIF) into the cytosol, which promotes activation of caspase 9, which, in turn, mediates activation of effector caspases (caspase 3, caspase 7 and caspase 6) (Czabotar et al., 2014)(Galluzzi et al., 2018). Effector caspases, in turn, mediate apoptotic such as DNA fragmentation, phosphatidylserine exposure and the formation of apoptotic bodies.

Extrinsic apoptosis is initiated when members of the tumor necrosis factor receptor (TNFR) family (ex. FAS or TNF receptor) are ligated (for example by FasL, TNF or TRAIL) at the plasma membrane. Ligation of the death receptors leads to the formation of multiprotein complex at the receptor's cytoplasmic tail called death-inducing signaling complex (DISC). DISC operates like a molecular platform mediating the activation of caspase 8. Caspase 8 activates executioner caspases and proteolytic cleavage of Bid protein to generate truncated Bid (tBid), which engages the mitochondrial apoptotic pathway by activating BAK and BAX (Czabotar et al., 2014)(Galluzzi et al., 2018).



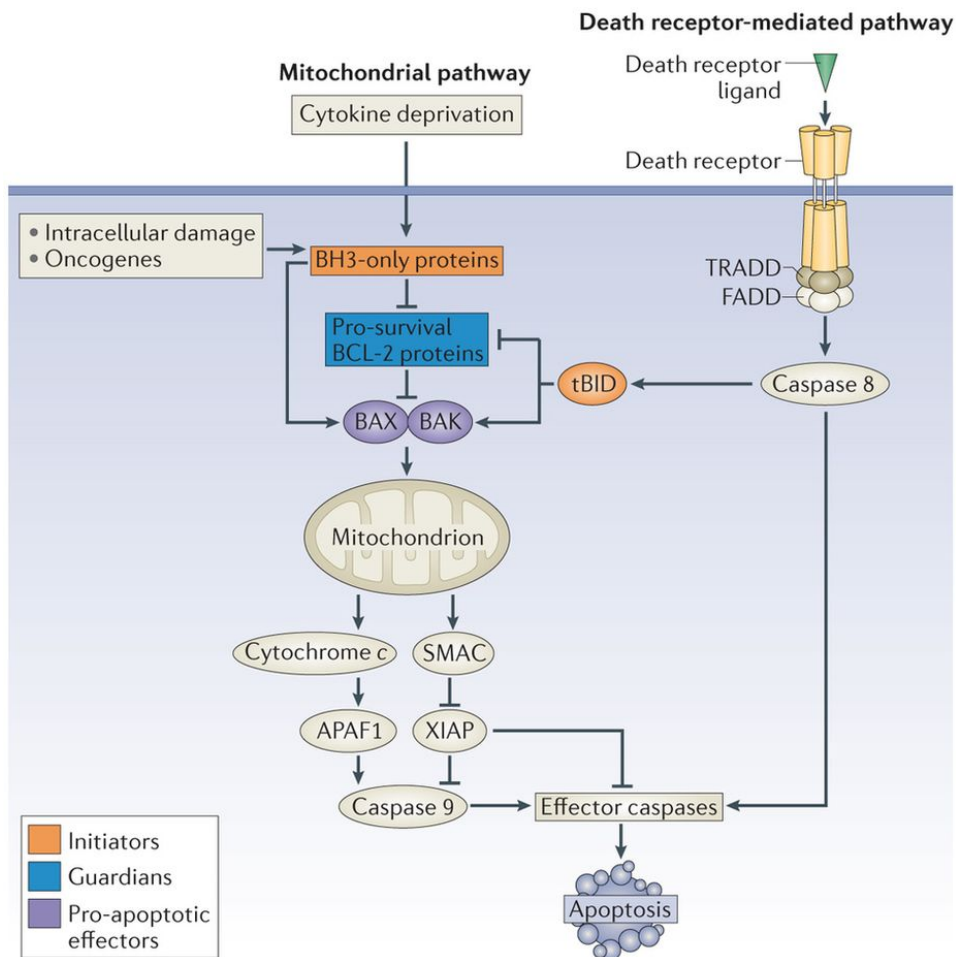
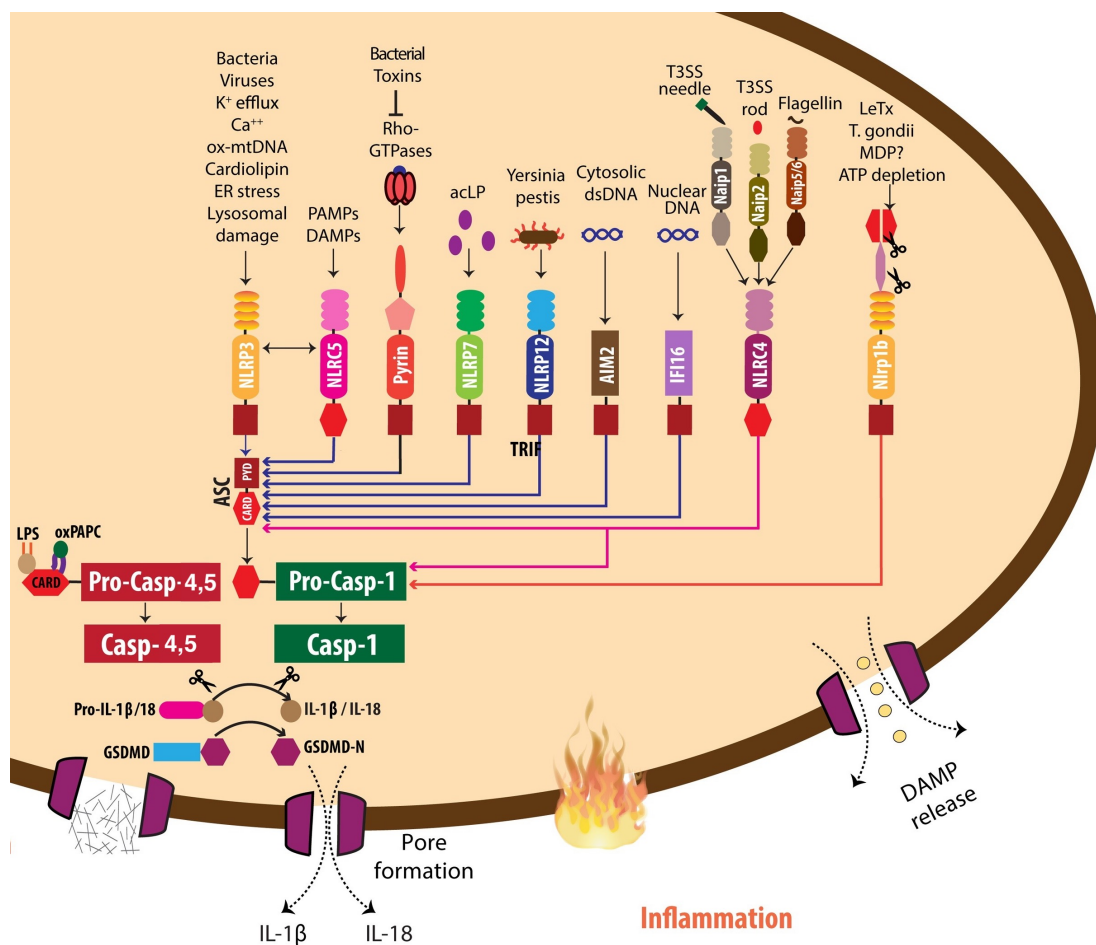


Figure 13. Basic apoptosis molecular pathways. Adapted from (Czabotar et al., 2014)

## Pyroptosis

In contrast to apoptosis, pyroptosis is a highly immunogenic form of cell death that takes place in response to recognition of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (such as  $K^+$  efflux) by the pattern recognition receptors (PRRs) (Kunzelmann, 2016). PRRs then assemble into multiprotein signaling complexes, called inflammasomes, and recruit adaptor protein apoptosis-associated speck-like protein containing C-terminal CARD (ASC), which then recruits and activates caspase-1 or

8 via their CARD domain (Songane et al., 2018) (see below) (Figure 14). These caspases cleave IL-18 and IL-1 $\beta$  to their mature forms. They also cleave and activate gasdermin D, which translocates to the inner leaflet of the PM generating a pore that leads to the secretion of mature IL-18 and IL-1 $\beta$ , K<sup>+</sup> efflux (Gutierrez et al., 2017), PM permeabilization and cell lysis (Galluzzi et al., 2018)(Jorgensen and Miao, 2015). The rapid rupture of the PM releases the cytoplasmic contents into the environments. DNA damage accompanies pyroptosis, similarly to apoptosis. However, there is little DNA fragmentation and, although chromatin condensation is observed, the nucleus remains intact (Table 2) (Jorgensen and Miao, 2015).



**Figure 14. Diversity of inflammasomes and their known activating signals. Adapted from (Songane et al., 2018)**

**Table 2. Morphological and molecular determinants of pyroptosis vs apoptosis. Adapted from (Jorgensen and Miao, 2015)**

Characteristic	Pyroptosis	Apoptosis
Inflammatory versus non-inflammatory	Inflammatory	Non-inflammatory
Lytic versus non-lytic	Lytic	Non-lytic
Initiator caspase	Caspase-1/4/5/11	Caspase-2, 8, 9, 10
Effector caspase	None	Caspase-3, 6, 7
DNA damage		
Laddering	No	Yes
TUNEL stain	Yes	Yes
ICAD cleavage	No	Yes
Chromatin condensation	Yes	Yes
Nucleus intact	Yes	No
Plasma membrane pore formation	Yes	No
PARP cleavage	No	Yes
AnnexinV staining	Yes	Yes

## ***Necroptosis***

Necroptosis is a regulated form of cell death that resembles necrosis. Therefore, morphological features of necroptosis are: cell swelling, rapid PM permeabilization, intact nuclei and the absence of caspase activation (Moreno-Gonzalez et al., 2016, p.)(Kunzelmann, 2016). Necroptosis can occur in response to signaling through the TNF receptor family, to triggering of PRR receptors and in response to IFN signaling (Figure 15). Key molecular events of necroptosis are association and autophosphorylation and transphorylation of kinases RIPK1 and RIPK3 and phosphorylation of MLKL (Tait et al., 2014) (Moreno-Gonzalez et al., 2016, p.)(Berghe et al., 2014). These events are accompanied by ROS production by the mitochondria (Berghe et al., 2014). Activated RIPK3 and MLKL are targeted to the PM (Moreno-Gonzalez et al., 2016, p.). MLKL oligomerizes and either form PM pores directly leading to Ca<sup>2+</sup> influx-dependent cell lysis or engages ion channels leading to influx of Na<sup>+</sup> or Ca<sup>2+</sup> ions triggering cell death (Moreno-Gonzalez et al., 2016, p.)(Berghe et al., 2014).

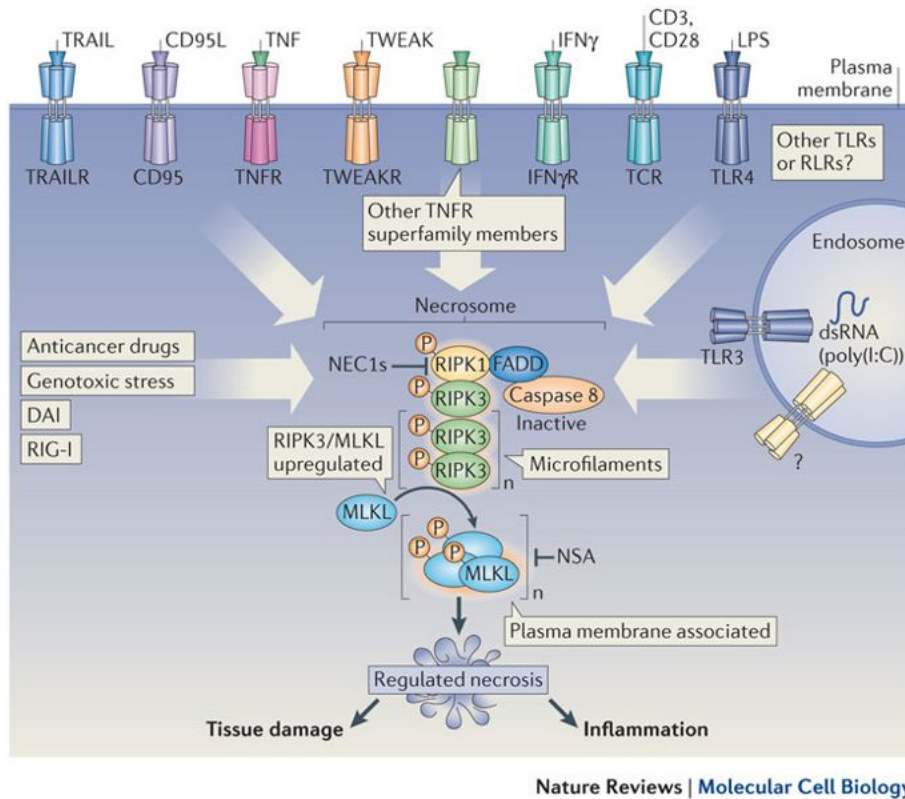


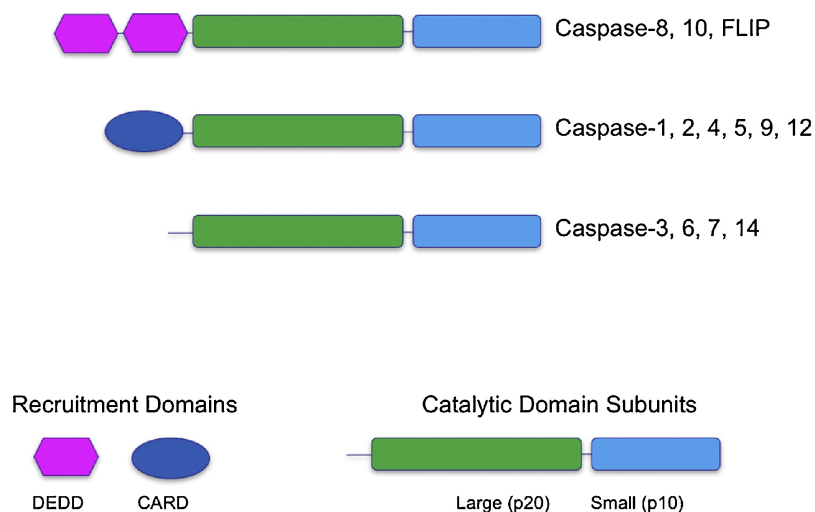
Figure 15. Triggers and central mechanism of necroptosis. Adapted from (Berghe et al., 2014)

## MOLECULAR PLAYERS OF CELL DEATH

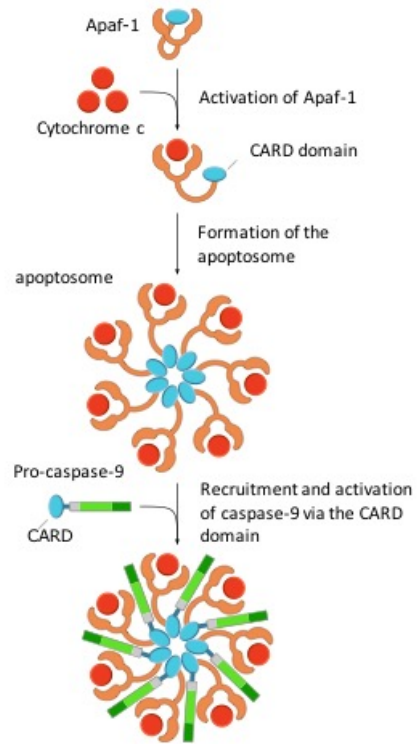
### *Caspases: initiators and effectors of cell death*

Caspases are a cysteine-dependent aspartate-specific proteases. They are produced as inactive zymogens and are activated in response to inflammatory and death signals. All caspases contain a C-terminal effector protease domain and some (caspases 1, 2, 4, 5, 9, 12) also contain an N-terminal caspase activation and recruitment domain (CARD) (Figure 16). Caspases 8 and 10, on the other hand, contain death effector domain (DED) at the N-terminus. Caspases are generally classified into initiator (caspase-2, 8, 9, 10) and effector classes (caspase-3, 6, 7) (Table 2). Initiator caspases are able to auto-activate and mediate, via cleavage, the activation of effector caspases, which then activate mediators of apoptosis (Man and Kanneganti, 2016).

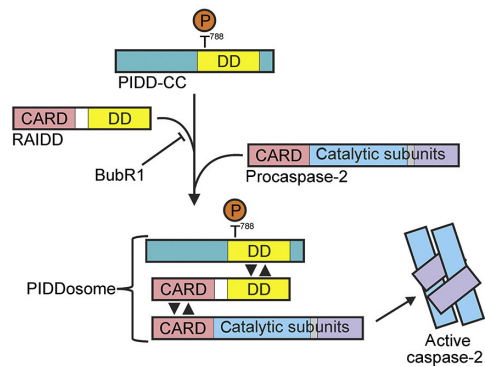
Zymogens of caspases 8 and 10 are inert monomers, which are recruited to the high molecular weight oligomeric molecular complexes such as death-inducing signaling complex (DISC) via their DED domain, where they are activated by dimerization and auto-cleavage (Songane et al., 2018). Similar mechanism is thought to operate for the activation of CARD-containing caspases. These caspases are also recruited into oligomeric protein complex via the interaction of their CARD domain with the CARD domain of the recruitment proteins. For example, caspase-9 is recruited via its CARD to interact with the CARD of Apaf-1 polymers in the apoptosome (Figure 17) (Ramirez and Salvesen, 2018). Induced proximity of caspase-9 monomers results in their dimerization and auto-cleavage between large and small subunits of the catalytic domain, which results in its activation. Caspase-1 is activated by its recruitment via the CARD domain to the inflammasome macromolecular complex. Similarly, caspase-2 interacts via its CARD domain with the CARD of RAIDD protein in the PIDDosome (Tinel and Tschopp, 2004) (Figure 18). Caspases 4 and 5 oligomerize and auto-activate via the CARD domain upon binding to bacterial lipopolysaccharide (LPS) (Ramirez and Salvesen, 2018). Zymogens of the effector caspases-3, 6 and 7 are dimers and their activity is inhibited by a linker separating small and large subunits of the catalytic domain (Figure 16). Activation of these caspases proceeds via their proteolytic cleavage by the initiator caspases through the cleavage of the linker to separate large and small subunits of the catalytic domain, which then assemble for full activity (Shi, 2004).



**Figure 16. Functional domains of caspases. Adapted from (Ramirez and Salvesen, 2018).**



**Figure 17. Recruitment and activation of caspase-9 via the CARD domain in the apoptosome. Adapted from Wikipedia Commons.**



**Figure 18. Activation of caspase 2 in the PIDDosome. Adapted from (Miles et al., 2017)**

The function of each caspase seems to be multidimensional albeit being not well characterized. Caspases-2, 3 and 6-10 are thought to activate apoptosis death pathways. Caspase-1 is thought to be activated primarily in the association with inflammasomes. This caspase mediates cleavage of IL-1 $\beta$  and IL-18 cytokines into their mature forms, although caspase-1 independent cleavage of these cytokines via is now known (Man and Kanneganti, 2016). Caspases-4 and 5 are also implicated in the non-classical inflammasome activation and initiation of pyroptosis pathway (Man and Kanneganti, 2016) by direct recognition of microbial products, activation and cleavage of IL-1 $\beta$  and Gasdermin D (Songane et al., 2018). Caspase-2 was also recently reported to be activated downstream of the inflammasome (Bronner et al., 2015) although it is most known to be activated in response to DNA damage to mediate apoptosis or cell cycle arrest (Olsson et al., 2015). Caspase-8, together with caspase-10 initiates extrinsic apoptosis pathway (Songane et al., 2018). It is implicated in the activation of the NF $\kappa$ B signaling pathway and, similarly to caspases 4 and 5, the cleavage of IL-1 $\beta$  and IL-18. It has also been reported to block the inflammasome activity. Caspase-12 function in humans remains enigmatic because of high prevalence of inactive short form of caspase-12 in human population (Man and Kanneganti, 2016). So far, the active form of this caspase has been proposed to limit the activation of the inflammasome and caspase-1. Additionally, caspase-14, most enigmatic of all, so far has not been implicated in inflammasome activation or apoptosis and seems to be specialized in keratinization of the skin (Ramirez and Salvesen, 2018).

### ***The role of ion channels in cell death***

The cell tightly controls the intracellular levels of mono- and divalent ions (Figure 19). During health, Ca<sup>2+</sup>, Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup> and other ions regulate variety of cellular processes. Ca<sup>2+</sup>, for example, signals T cell activation, K<sup>+</sup> and Cl<sup>-</sup> regulate cell volume. It is not surprising that perturbation of intracellular ion homeostasis, therefore, was shown to accompany the process of cell death. Intracellular Ca<sup>2+</sup> homeostasis is controlled by Ca<sup>2+</sup> channels and pumps located in the plasma membrane (PM) and in the membranes of various organelles (Kondratskyi et al., 2015) (Kunzelmann, 2016). Ca<sup>2+</sup> overload of the cytoplasm stimulates mitochondrial Ca<sup>2+</sup> uptake which triggers opening of mitochondrial permeability transition pore (MPTP), activating

intrinsic apoptotic pathway. Cytoplasmic  $\text{Ca}^{2+}$  can also activate  $\text{Ca}^{2+}$ -dependent cysteine proteases, caplains, which cleave BID, BCL-2 and BCL-XL and trigger MOMP.

Cellular  $\text{K}^+$  and  $\text{Na}^+$  homeostasis are directly linked. While the cell ensures high intracellular  $\text{K}^+$  concentration (around 130mM),  $\text{Na}^+$  is continuously exported to results in 15mM intracellular concentration. This is achieved by the action of  $\text{Na}^+/\text{K}^+$  ATPase. In addition, multiple  $\text{Na}^+$  channels (similarly to  $\text{Ca}^{2+}$  channels) mediate rapid intracellular  $\text{Na}^+$  increase during membrane depolarization. An increase in intracellular  $\text{Na}^+$  occurs early in apoptosis (Kondratskyi et al., 2015). However, how  $\text{Na}^+$  is involved in molecular pathways of cell death is poorly understood.

Apoptotic volume decrease (AVD) is a hallmark and the initial step of apoptosis and is caused by  $\text{K}^+$  and  $\text{Cl}^-$  efflux.  $\text{K}^+$  leaves the cell through the activation of  $\text{K}^+$  channels (ex. Voltage-gated Kv, ATP-regulated  $\text{K}_{\text{ATP}}$ ,  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channels, etc) (Kunzelmann, 2016). Similarly,  $\text{Cl}^-$  leaves the cell through  $\text{Cl}^-$  channels (voltage gated,  $\text{Ca}^{2+}$ -activated, volume regulated anion, ligand gated and other channels). Loss of intracellular  $\text{K}^+$  is thought to favor activation of caspases and nucleases (Kondratskyi et al., 2015), although it was found not to be obligatory for apoptosis to take place (Börjesson et al., 2011). Moreover, increase in extracellular  $\text{K}^+$  and  $\text{Cl}^-$  concentration was reported to inhibit apoptosis (G. J. Thompson et al., 2001).



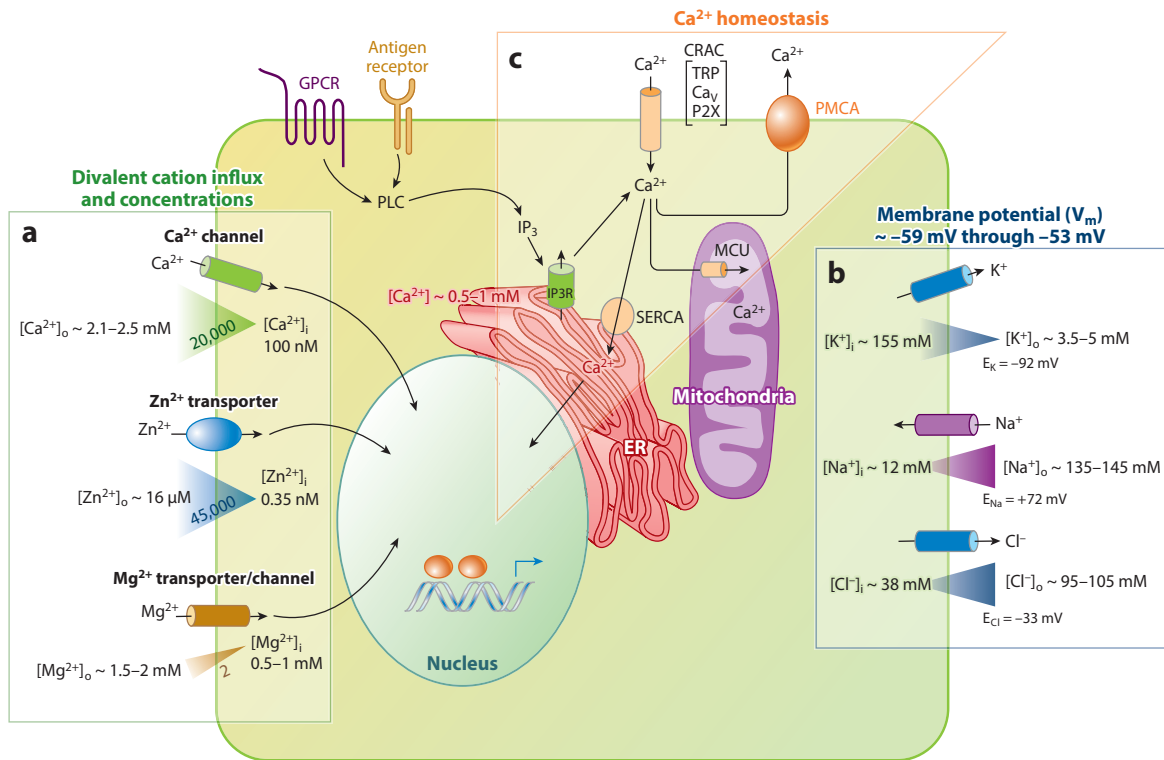


Figure 19. Intracellular homeostasis of various ions. Adapted from (Feske et al., 2015a)

## CELL DEATH IN HIV-1 INFECTION

The hallmark of HIV-1 infection is the depletion of CD4<sup>+</sup> T cells. Hence, the dogma in the field was, until recently, that the virus promotes cell death of infected cells. Indeed, this may be the case during acute phase of infection, when rapid viral replication leads to massive CD4<sup>+</sup> T cell depletion. During chronic infection, however, the contribution of direct viral cytotoxicity to cell death is limited as the percentage of cells actively producing the virus is lower (Finzi et al., 1997)(Embretson et al., 1993). This also supported by the lack of appreciable effect of latency reversal agents, that have not been shown to eliminate infected cells due to virus reactivation (Lehrman et al., 2005; Rasmussen et al., 2014; Rasmussen and Lewin, 2016; Routy et al., 2012). Additionally, apoptosis has been shown to occur predominantly in ‘bystander’ cells in lymphoid tissues and not in productively infected cells (Finkel et al., 1995). Viral persistence in the infected cell that survive even during cART tells us that the cell’s life/death balance during HIV-1 infection may be more complex than once appreciated.

Overall, it seems that the activation status of cells plays a role in the susceptibility to cell death during HIV infection. Like so, highly activated effector memory CD4<sup>+</sup> T cells are depleted fastest and first from gut mucosal sites (Grossman et al., 2006) and naïve T cells displaying resting phenotype are resistant to depletion in lymphoid tissues (Veazey et al., 2000). Additionally, it was shown that blood-derived CD4<sup>+</sup> T cell that display deeper resting state than lymphoid tissue derived cells are more resistant to pyroptosis despite carrying viral genetic material (Muñoz-Arias et al., 2015). Thus, infected naïve, central memory and stem cell like memory T cells displaying less activated phenotype could be less prone to these mechanisms of virally induced cell death than highly activated effector memory T cells, and thus persist longest hence supplying the reservoirs (Olvera-García et al., 2016)(Buzon et al., 2014).

On the molecular level, multiple mechanisms have been described to either favour death or survival of infected cells but how exactly the virus modulates these is unknown. *In vitro* activated, infected CD4<sup>+</sup> T cells have been reported to undergo integration-dependent cell death, which has been linked to the recruitment of DNA-PK (Cooper et al., 2013a). Activated productively infected T cells have also been shown to express activated caspase-3 (Doitsh et al., 2010) and undergo caspase-3 mediated cell death (Jekle et al., 2003). On the other hand, death of non-productively infected CD4<sup>+</sup> T cells have been reported to result from the detection of viral reverse transcription products by DNA sensor IFI16, which leads to caspase-1 activation thereby triggering pyroptosis (Doitsh et al., 2014, 2010; Monroe et al., 2014, p. 2).

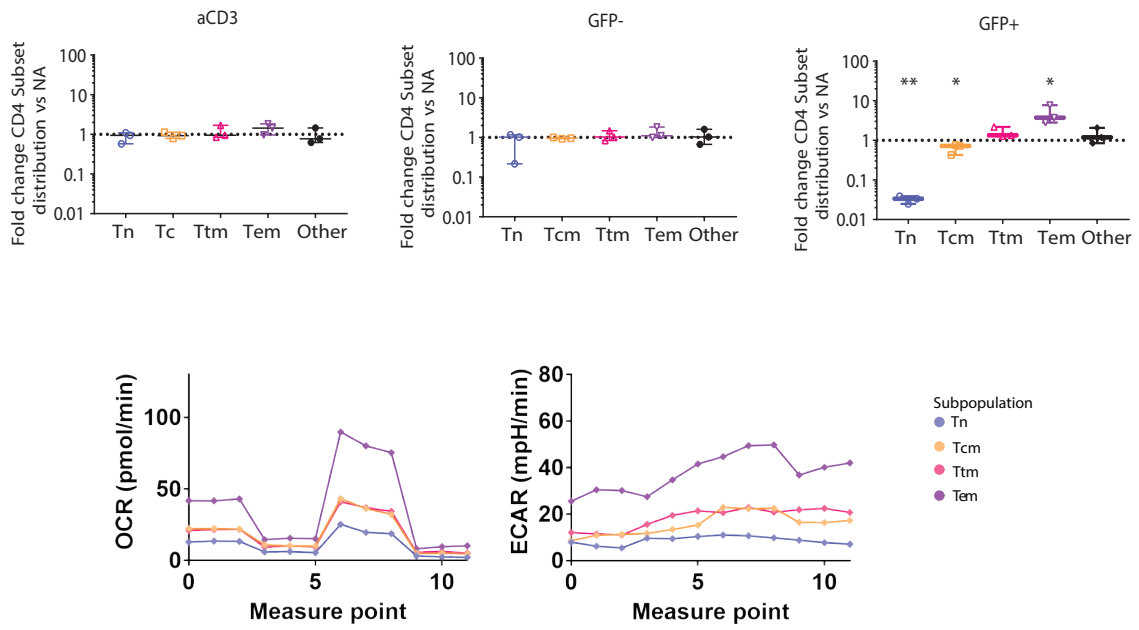
Viral proteins could have a dual role in modulating pro- and anti-survival signals. Productively infected cells were shown to be protected from Fas-mediated apoptosis by expression of Nef protein, which inhibits Fas signaling (Geleziunas et al., 2001)(Ohnimus et al., 1997). Nef was also shown to inactivate pro-apoptotic BAD protein hence rendering infected T cells more resistant to apoptosis (Wolf et al., 2001a). Env was observed to induce resistance to TRAIL-

induced apoptosis in macrophages (Swingler et al., 2007). On the other hand, viral proteins Tat (Westendorp et al., 1995), Env (Cicala et al., 2000), Vpr (Muthumani et al., 2002) and Nef (Muthumani et al., 2005; Xu et al., 1999) were shown to have an apoptotic effect *in vitro*. Thus, how the virus modulates cell death pathways remains an open question.

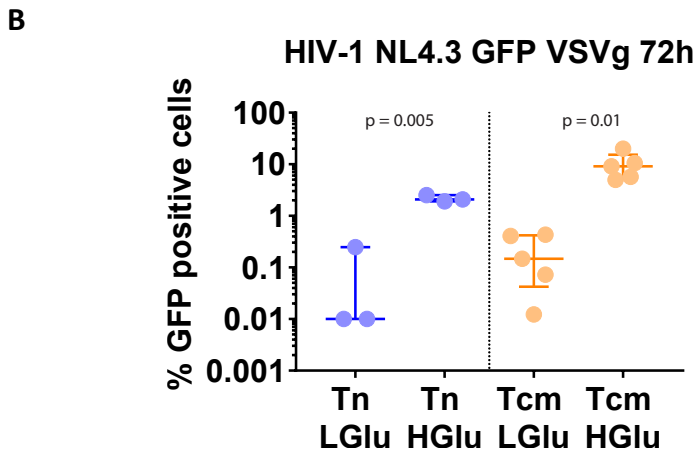
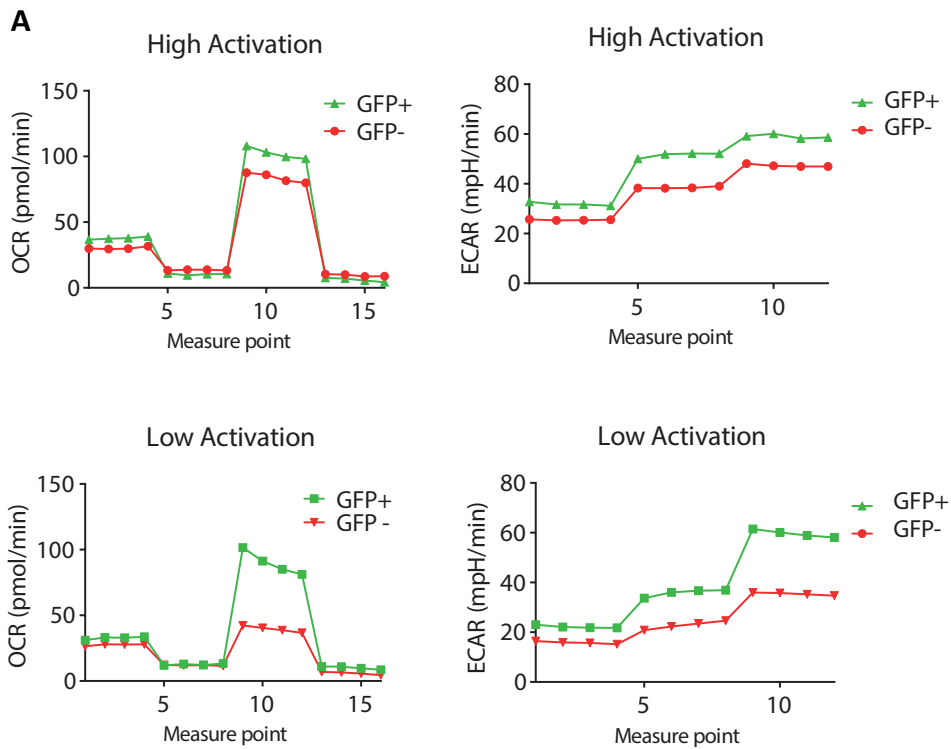
# HYPOTHESIS AND OBJECTIVES

## CONTEXT

In recent years, the lab of Dr. Saez-Cirion focused part of its efforts in understanding how the HIV virus establishes productive infection in CD4+ T cells as well as macrophages and DCs. One of the important questions that was addressed was how does intracellular environment of different CD4+ T cell memory subsets, which are known to contribute differently to the HIV reservoir, dictate their relative susceptibility to infection. Naïve CD4+ T cells are known to be resistant to the establishment of productive viral cycle, while memory subsets are permissive. It is well established that the activation plays a key role in cellular permissiveness to infection. Additionally, the role of CD4+ T cell metabolism is now beginning to be appreciated. Therefore, JC Valle-Casuso et al investigated activation and metabolic factors that could play a role in the establishment of productive infection in four CD4+ memory subsets: Tn, Cm, Tm and Em. The expression of metabolism-related genes was correlated to the degree of infection seen in different CD4+ T cell subsets. This work (see Annex 2) clearly showed the dependence of the viral replication on metabolic activity, especially glycolysis (but also OXPHOS), of each cell subset. The relative contribution of each memory subset (as compared to non-activated cells) directly mimicked the metabolic profile of that cell subset (Figure 20). This work further showed that the virus preferentially infects metabolically active cells within each subset and this was independent of cell activation and differentiation status (Figure 21A). Finally, inhibition of glycolysis by glucose starvation or glucose analog 2-deoxyglucose (2 DG) reduced viral infection via suppression of reverse transcription (Figure 21B).



**Figure 20. Relative contribution of CD4+ T cell memory subsets to infected cell pool and their respective metabolic profiles. Adapted from Valle-Casuso, submitted (Annex 2)**



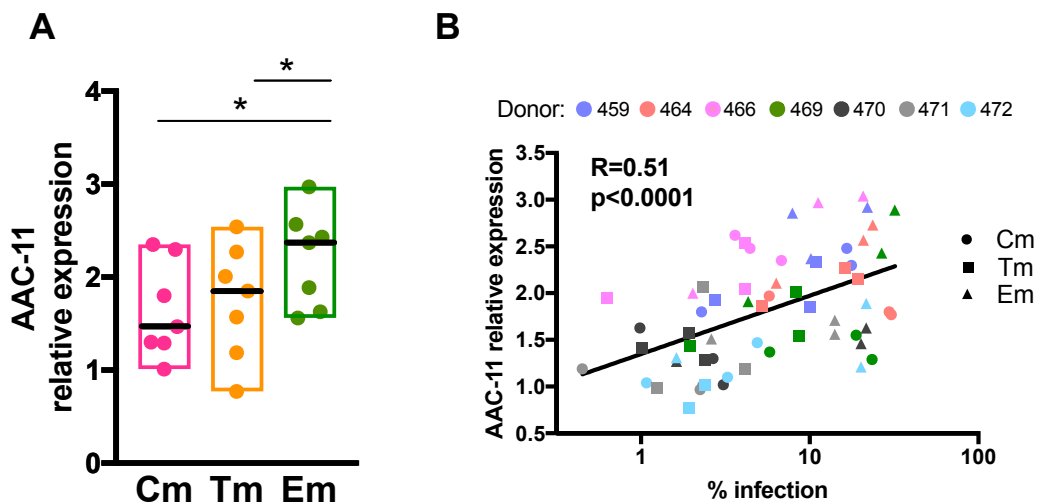
**Figure 21. HIV-1 preferentially infects metabolically active cells irrespective of their activation status and differentiation stage. A) HIV-1 infected cells (GFP+) display higher metabolic activity (maximal respiratory capacity) and glycolysis than exposed non-infected (GFP-) cells. B) HIV-1 infected cells are preferentially contained within high glucose consuming cell population (HGlu) vs within low glucose consuming cells (LGlu)**

This work indentified a clear relationship between the ability of different CD4+ T cell memory subsets to be infected and their metabolic status. Yet another important question is how HIV-1 susceptible cells survive *in vitro* cytopathic effect of the virus and other cell death-triggering mechanisms operating *in vivo* to establish productive viral infection leading to the formation of the reservoir? CD4+ T cell subsets vary in their half-life *in vivo* (Gattinoni et al., 2011)(Lugli et al., 2013)(Mahnke et al., 2013) and it is interesting that HIV-1 persists in permissive subsets that display long-term survival such as Cm cells (Olvera-García et al., 2016). We thus wondered if the intrinsic anti-apoptotic pathways expressed by CD4+ memory T cells (Gattinoni et al., 2011)(Lugli et al., 2013)(Mahnke et al., 2013) could be responsible for the survival of these cells upon infection leading to their dominant contribution to HIV-1 reservoirs (Chomont et al., 2009b). If so, what would be these pathways and is it possible to counteract them to prevent survival of HIV-1 target cells?

JC Valle-Casuso et al analyzed gene expression of factors implicated in activation, metabolism and cell death and correlated their expression to the proportion of infection of CD4+ T cells. Among factors implicated in cell death pathways (ex. FAS, E2F1, IFI16, CASP3, MTOR, CASP8, BCL11B, IL17R), AAC-11 expression increased with the state of differentiation of memory CD4+ T cell subsets (Cm<Tm<Em) and correlated significantly with infection in each subset (Figure 22A and B).

AAC-11 attracted our attention due to its described role in the protection of cancer cells against apoptosis in the conditions of metabolic stress. HIV-1 targets highly glycolytic cells for productive infection (Loisel-Meyer et al., 2012b)(Hegedus et al., 2014b; Hollenbaugh et al., 2011b)(Palmer and Crowe, 2016)(Valle-Casuso, submitted; see Annex 2), as these cells provide the right combination of metabolic substrates and cell factors for the progression of its life cycle. At the same time, long-term persistence of HIV-1 infected cells *in vivo* indicates that HIV-1 could also be targeting cells with upregulated survival pathways, as was already shown for Cm cells (Olvera-García et al., 2016). These two aspects parallel the hallmarks of cancer cells, which are known to be, on one hand, high level of glycolysis (Warburg effect), and on the other hand, resistance to apoptosis.

AAC-11 was first identified in mice and was shown to protect cells from apoptosis in serum deprivation grown conditions (Tewari et al., 1997). It is overexpressed in many cancers (Koci et al., 2012) and has been shown to support cancer cell survival in the conditions of growth factor withdrawal (Tewari et al., 1997). Its expression is associated with poor prognosis in non-small lung and cervical cancers (Cho et al., 2014; Sasaki et al., 2001; Wang et al., 2010). AAC-11 contains several protein interaction domains, including leucine zipper (LZ) domain (Han et al., 2012) and was shown to inhibit apoptotic effectors such as E2F1 (Morris et al., 2006), Acinus (Rigou et al., 2009) and caspase-2 (Imre et al., 2017). AAC-11 prevents apoptotic Acinus-mediated DNA fragmentation by interacting with it via its LZ domain (Rigou et al., 2009). It has also been reported to inhibit caspase-2 activity via its direct binding to caspase recruitment domain (CARD) and prevention of caspase-2 dimerization and activation (Imre et al., 2017). Given AAC-11's anti-apoptotic function, we wondered if HIV-1 targets cells that rely on AAC-11 pathway for survival.



**Figure 22. AAC-11 gene expression in CD4+ T cell memory subsets and its correlation with infection. A. AAC-11 gene expression increases with CD4+ T cell memory differentiation (Cm<Tm<Em) B. Correlation of AAC-11 gene expression at the time of infection in different CD4+ T cell memory subpopulations with the percent infected cell in that subset at day 3 of infection.**



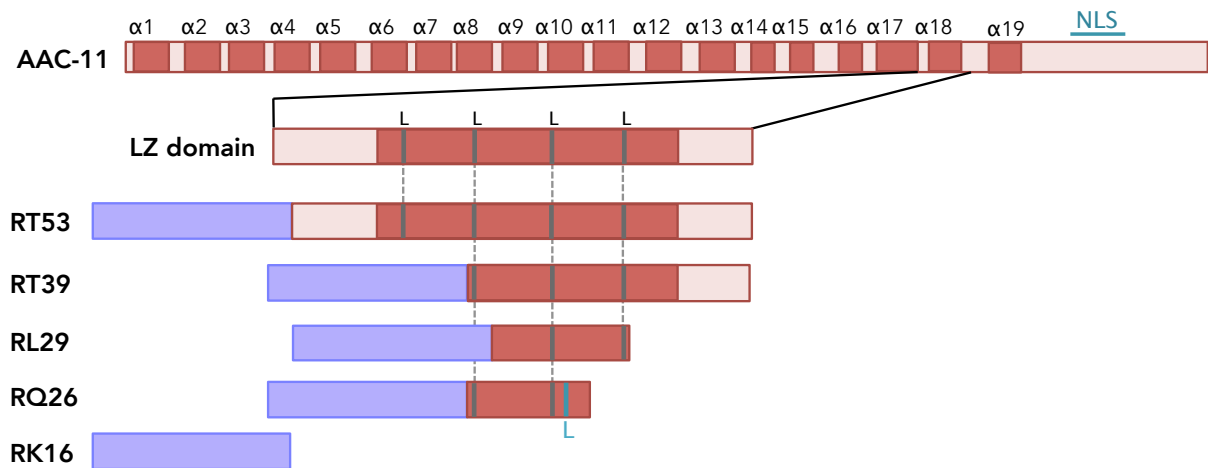
## HYPOTHESIS

HIV-1 preferentially targets cells programmed for survival via the high expression of AAC-11. This factor, therefore, could represent a target of vulnerability of HIV infection, which can be exploited by counteracting its anti-apoptotic activity to selectively eliminate cells supporting the establishment of HIV-1 infection.

## APPROACH

In order to counteract AAC-11 activity we used a panel of peptides designed and kindly provided to us by the team of Dr. Jean-Luc Poyet at Hôpital Saint Louis. The design of these peptides was based on the LZ domain of AAC-11 fused to penetratin peptide of *Drosophila melanogaster* (here designated as RK16) (Figure 23). Penetratin was shown to spontaneously cross cellular membranes and is used as an intracellular delivery method for various molecules (Derossi et al., 1998). AAC-11-derived peptides were designed to competitively inhibit AAC-11 interaction with its partners via its LZ domain (Jagot-Lacoussiere 2016). Dr. Poyet's team already successfully used RT53, the peptide that spans the complete length of the LZ domain and is fused to penetratin (Figure 23), to antagonize the survival of cancer cells both *in vitro* and *in vivo* in a murine model of melanoma (Jagot-Lacoussiere et al., 2016).

We thus tested if AAC-11 derived peptides would counteract the survival of HIV-1 target cells. To this end, we treated infected and non-infected cells with these peptides and determined their effect on the survival of HIV-1 infected cells.



**Figure 23. Schematic of AAC-11 derived peptides.**

## OBJECTIVES

1. To determine if AAC-11-derived peptides are effective at eliminating cells susceptible to productive HIV-1 infection or inhibiting infection
2. To determine how the viral life cycle is impacted
3. To establish the characteristics of the cell subset affected by AAC-11-derived peptides
4. To determine the mechanism of action of AAC-11-derived peptides

**CHAPTER 1:**  
**AAC-11 DERIVED PEPTIDES INDUCE DEATH OF CD4+ T CELLS**  
**SUSCEPTIBLE TO HIV-1 INFECTION**

Primary article, submitted

1 **AAC11 derived peptides induce death of CD4+ T cells susceptible to HIV-1 infection**

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13

14 **ABSTRACT**

15 Despite viral cytotoxic effect classically associated with *in vitro* infection and massive depletion of viral  
16 target cells during infection *in vivo*, HIV-1 successfully establishes infection and persists in its  
17 reservoirs. However, how productively infected cell survive and persist remains to be understood.  
18 Here we propose that HIV-1 relies on Anti-apoptotic clone 11 (AAC11) anti-apoptotic pathway for the  
19 establishment of productive infection in CD4+ T cells. We observed the expression of AAC11 to  
20 increase with progressive CD4+ T cell memory differentiation in association with the expression of cell  
21 cycle, activation and metabolism genes and to correlate with susceptibility to HIV infection.  
22 Antagonism of AAC11 survival pathway with peptides derived from its sequence lead to cell death of  
23 HIV-1 susceptible target cells and selection of cells resistant to infection. The peptides caused  
24 preferential elimination of activated and metabolically active cells, in particular effector and  
25 transitional CD4+ T cell memory subsets, and their activity was at least in part dependent on caspase  
26 2 activation. Thus, our results provide a proof of concept that the selective targeting of the survival  
27 pathways used by HIV-1 to persist is a possible approach to antagonize seeding of HIV-1 CD4+ T cell  
28 reservoirs.

29  
30 **INTRODUCTION**

31 Human immunodeficiency virus type 1 (HIV-1) is a persistent viral infection that has claimed millions  
32 of lives around the globe. The era of combination anti-retroviral therapy (cART) has extended life  
33 expectancy of people living with HIV and improved their quality of life. However, persistence of viral  
34 reservoirs remains a major challenge as they are at the origin of viral rebound if therapy is interrupted  
35 (Davey et al., 1999; García et al., 1999; Harrigana et al., 1999). Therefore, recent efforts of the scientific  
36 community have been focused on the precise characterization of HIV-1 reservoirs in the hope of  
37 designing new treatment strategies for their prevention and/or elimination.

38

39 Memory CD4+ T cell subsets are thought to be the major contributor to the persistent HIV-1  
40 reservoir under cART (Barton et al., 2016). Among these, central memory CD4+ T cells were shown  
41 to be important due to their self-renewal ability and long-term survival, while transitional and  
42 effector memory cells were shown to sustain the reservoir by homeostatic proliferation (Chomont,  
43 Nat Med, 2009) (Heiner, 2017). The seeding of the HIV reservoir in different CD4+ T cell subsets is  
44 first determined by their relative susceptibility to HIV-1 infection. Naïve CD4+ T cells are relatively  
45 resistant to infection and susceptibility to HIV increases with T cell differentiation and activation. We  
46 have recently described how the infectability of CD4+ T cell subsets is related to their metabolic  
47 activity (Valle-Casuso, submitted). Numerous reports have shown that immunometabolism is a  
48 critical element in the regulation of T cell differentiation, survival and function (Almeida et al., 2016).  
49 Upon antigenic stimulation T cells upregulate metabolic fluxes to provide the energy necessary to  
50 support cellular processes and to increase the pool of substrates necessary for building proteins,  
51 lipids, nucleic acids and carbohydrates. We and others have shown that this metabolically rich  
52 environment is necessary for the establishment of both productive and latent HIV infection (Valle-  
53 Casuso, submitted) (Hegedus et al., 2014; Hollenbaugh et al., 2011), as it is also the case for other  
54 infections (Heaton et al., 2010; Manel et al., 2003; McArdle et al., 2012).

55

56 The survival of infected cells, including to the cytopathogenic effects of the virus, is another critical  
57 characteristic for the persistence of the HIV reservoir that is not well understood. In our previous  
58 study (Valle Casuso, submitted) we found that, together with several other genes associated with  
59 cell metabolism, the anti-apoptosis clone 11 (AAC11) [also known as apoptosis inhibitor 5, API5] was  
60 significantly correlated with infection in different subsets of memory CD4+ T cells. AAC11 is  
61 overexpressed in many cancers (Koci, 2012) and allows cancer cell survival in conditions of metabolic  
62 stress (Kim et al., 2000). Its expression is associated with poor prognosis in non-small lung and  
63 cervical cancers (Wang, 2010; Sasaki, 2001; Cho, 2014). Although the mechanisms associated with its  
64 antiapoptotic activity have not been clearly elucidated, AAC11 contains several protein interaction

65 domains, including a leucine zipper (LZ) domain (Han, 2012), and has been proposed to repress  
66 apoptotic effectors such as E2F1 (Morris, 2006), Acinus (Rigou, 2009) and caspase-2 (Imre, 2017).  
67 We hypothesized that, similarly to its role in cancer, the antiapoptotic activity of AAC11 could  
68 contribute to the survival of metabolically active cells that are preferential targets of HIV, hence  
69 representing a potential target of vulnerability in infection.

70

71 In order to antagonize the activity of AAC11 we used a panel of synthetic peptides based on the LZ  
72 domain sequence of AAC11 to impair the interaction of AAC11 with potential pro-apoptotic  
73 partners. Some of these peptides were previously shown to be cytotoxic to cancer cells both *in vitro*  
74 and *in vivo* in a mouse model of melanoma (Jagot-Lacoussiere, 2016). We found that AAC11  
75 peptides blocked HIV infection through the preferential killing of HIV-1 'infectable' cells thus serving  
76 as a proof of concept that selective elimination of HIV-1 targets is a possible therapeutic strategy in  
77 tackling infection of CD4+ T cells.

78

79

80 **RESULTS**

81 **AAC11 derived peptides display anti-viral activity against diverse HIV-1 viral strains and SIV**

82 We previously analyzed the susceptibility of memory CD4+ T cell subpopulations (central (Cm),  
83 transitional (Tm) and effector (Em) memory) to infection with single cycle HIV NL4.3  $\Delta$  Nef  $\Delta$  env GFP  
84 particles pseudotyped with VSVG envelope. We also analyzed the expression, at the time of  
85 infection, of a panel of 96 genes related to T cell differentiation, function and survival as well as  
86 restriction and HIV facilitating factors (Valle-Casuso, submitted). Among other genes, we found the  
87 expression of anti-apoptotic factor AAC11 to increase progressively with the stage of differentiation  
88 of memory CD4+ T cells (Cm<Tm<Em) and to correlate with infection in these subsets (Fig 1A,B). The  
89 expression of AAC11 was strongly correlated with the expression of multiple genes related to cell  
90 metabolism (e.g. SLC1A5, MTOR, HIF1A, GUSB, GAPDH), cell cycle (e.g. RHOA, RB1, MAPK1, MKI67)  
91 and genes well known to be necessary for different steps of the HIV replication cycle (RRM2,  
92 SAMRCB1, DDB1, CFL1, ACTB, CDK9, NFKB1) (Fig 1C). We hypothesized that the AAC11 antiapoptotic  
93 pathway is upregulated in highly metabolically active cells, which offer an optimal environment for  
94 HIV replication (Valle-Casuso, submitted), and may facilitate HIV persistence by allowing the survival  
95 of infected cells.

96

97 To explore this possibility we sought to antagonize the action of AAC11 using a panel of synthetic  
98 peptides derived from the LZ domain located in the alpha helix 18 of AAC11 (Han, 2012) fused to a  
99 cell penetrating sequence of antennapedia protein of *Drosophila melanogaster* [also commonly  
100 known as penetratin] (RK16 here) (Derossi, 1998) (Fig 2A). These peptides act as competitive  
101 inhibitors of protein-protein interactions, can prevent AAC11 antiapoptotic activities and have  
102 shown anti-tumor activities *in vitro* and *in vivo* (Jagot-Lacoussiere, 2016). We applied these peptides  
103 to activated primary human CD4+ T cells (Sáez-Ciri3n et al., 2011) after spinoculation with HIV-1 and  
104 measured infection 3 days later by flow cytometry (Fig 2B). AAC11-derived peptides reduced HIV  
105 infection to various degrees in a concentration-dependent manner (Fig 2C). Shorter peptides,



106 constructed from progressively smaller regions of AAC11 LZ domain were less effective at inhibiting  
107 infection (Fig 2C, D). As expected, RK16 did not show any anti-viral effect even at the highest  
108 concentration tested. We observed a rapid drop in the proportion of infected cells at 6 $\mu$ M peptide  
109 concentration for RT53 and RT39, peptides exhibiting the strongest anti-viral effect. At this  
110 concentration, RT53 was most potent at suppressing infection (Fig 2D), indicating that the entire  
111 length of the LZ domain of AAC11 was necessary for full peptide potency.

112

113 Moreover, we saw that RT53 was able to inhibit infection of diverse HIV-1 isolates (Fig 3). Both R5  
114 (Bal) and X4 (NL4.3) viruses as well as primary isolates (BX08, DH12, 132w) and single cycle VSVG-  
115 pseudotyped HIV-1 particles were inhibited, albeit less efficiently for the latter. RT53 was also able  
116 to inhibit *in vitro* infection of human CD4+ T cells with simian immunodeficiency virus SIV<sub>mac251</sub> and  
117 viral spread from splenic CD4+ T cells of Cynomologus macaques previously infected with SIV<sub>mac251</sub>.  
118 This is coherent with the high conservation of AAC11 across species (Morris et al., 2006) and 100%  
119 homology between humans and macaques. In contrast, RK16 alone had no inhibitory effect. Overall,  
120 our results show that AAC11 derived peptides were able to impair HIV-1 and SIV infection of CD4+ T  
121 cells *in vitro* or spread from *in vivo* infected CD4+ T cells.

122

### 123 **Blockage of HIV infection by AAC11-derived peptides is associated with cell death**

124 Since our goal was to use the peptides to antagonize the anti-apoptotic activity of AAC11, we next  
125 evaluated the amount of cell death in our cultures. Indeed, we saw that all peptides caused various  
126 rates of cell death (Fig S1A). Moreover, we found that the proportion of cell death caused by  
127 different peptides in different donors was negatively correlated with the proportion of HIV infected  
128 cells in the cultures (Fig S1B). As before, RT53 was most potent at causing cell death. Interestingly,  
129 RT53 induced similar death rates in cells infected with various HIV-1 strains and in non-infected cells  
130 (Fig 3B). This suggested that the peptides caused cytotoxicity of cells with particular intrinsic anti-  
131 apoptotic program that were also targeted by HIV for infection. Overall, these results supported the

132 idea that HIV-1 preferentially infected cells that rely on AAC11-survival pathway. Hence,  
133 antagonizing this survival pathway may lead to selective elimination of HIV-1 targets.

134

### 135 **Cell death induced by AAC11-derived peptides is associated with the activation of caspase 2**

136 We then decided to investigate the mechanism of RT53-mediated effect. We started by evaluating  
137 the kinetics of RT53 action. We used “real time” flow cytometry to read out changes in several  
138 molecular indicators associated with cell death as a function of time (Elliott et al., 2006) (Fig 4A). We  
139 found that treatment of activated CD4+ T cells with RT53 induced a sharp and rapid increase in the  
140 surface levels of phosphatidylserine (PS), as determined by binding to annexin V-FITC. In a subset of  
141 RT53-treated CD4+ T cells, we also observed a decrease in the intracellular levels of K<sup>+</sup> ion, the efflux  
142 of which has been linked to the activation of the cell death machinery (Bortner JBC 1997; McCarthy  
143 Cell Death Different 1997; Yu, 2003). These events were accompanied by the increase in DNA  
144 labelling with 7-Aminoactinomycin D (7-AAD), a classical marker for dead cells. Cell death peaked at  
145 30 minutes after treatment with the peptides in both non-infected and infected cells, with no  
146 significant further increase at later time points (Fig 4B). This rapid action indicated that RT53 could  
147 act as a molecular switch for a repressed cell death-triggering cellular factor that was strongly  
148 enriched in a subset of cells.

149

150 We have found that the expression levels of AAC11 correlated with gene expression of caspase 3 (Fig  
151 1B). We thus evaluated the activation of caspase 3 and other caspases, known activators and  
152 executioners of cell death. Although we did not see significant activation of caspase 1 or caspases 3  
153 and 7, we saw a strong activation of caspase 2 in cells treated with RT53 (Fig 4C). Activation of  
154 caspase 2 was maximal within the first hour after treatment and decreased afterwards (Fig 4D), in  
155 agreement with the kinetics of cell death triggered by the peptides. To further link cell death and  
156 viral inhibition we sought to revert peptide-induced cytotoxicity by antagonizing intracellular K<sup>+</sup>  
157 efflux with increasing KCl concentrations in the culture medium (Thompson et al., 2001). Non-

158 infected CD4+ T cells or cells that had been pre-infected with VSVG-pseudotyped HIV particles were  
159 incubated with RT53 or RK16 for 5 hours in presence of increasing extracellular K<sup>+</sup> concentration,  
160 from around 5mM in control condition up to 100 mM upon addition of increasing amounts of KCl  
161 (Fig 4E). We observed a KCl concentration-dependent decrease in RT53-mediated cell death, relative  
162 to RK16 or control condition, which was accompanied by a progressive increase in the proportion of  
163 infected CD4+ T cells recovered at the end of the culture. No differences were observed in the rates  
164 of cell death and the percentage of infected cells between RT53 and RK16 or control when the  
165 cultures were done in the presence of 100 mM of KCl, although we should notice that at this  
166 concentration the rates of infection were overall lower than in CD4+ T cells cultured in the absence  
167 of extra KCl (Fig 4E). Overall, our results confirmed that RT53 induced cell death in a subset of CD4+  
168 T cells and that this cytotoxic effect was directly related to the antiviral effect of the peptide.

169

#### 170 **RT53 preferentially depletes effector and transitional memory CD4+ T cell subpopulations**

171 We next assessed the characteristics of the CD4+ T cells that were sensitive to the cytotoxic action of  
172 RT53. Our results suggest that the AAC11 survival pathway was upregulated with CD4+ T cell  
173 differentiation (Fig 1A). We thus explored if RT53 treatment changed the distribution of CD4+ T cell  
174 subsets. When compared to non-treated or RK16-treated conditions, RT53 treatment changed the  
175 relative contribution of CD4+ T cell subsets to the total pool of CD4+ T cells (Fig 5A, Fig S2). Naïve  
176 CD4+ T cells were enriched upon treatment with RT53 while the more differentiated Tm and Em  
177 CD4+ T cells were depleted. Although activation *in vitro* changed somewhat the relative contribution  
178 of CD4+ T cell subsets to the pool of CD4+ T cells, RT53 had a similar impact on CD4+ T cell subsets  
179 when we used non-activated or *in vitro* activated cells (Fig 5A). These results show that the different  
180 susceptibility of the CD4+ T cell subsets to the action of the peptides was related to the intrinsic  
181 characteristics of each subset rather than provoked by cell activation. In agreement with other and  
182 our own previous results (Valle-Casuso, submitted)(Roederer et al., 1997; Schnittman et al., 1990;  
183 Spina et al., 1997), naïve CD4+ T cells were highly resistant to infection with both VSVG pseudotyped

184 HIV-1 particles and HIV-1 Bal. Susceptibility to infection increased with T cell differentiation, with Em  
185 and Tm cells showing the highest infection levels (Fig 5B). Thus, our results confirmed that RT53  
186 preferentially targeted differentiated memory cells with heightened susceptibility to HIV infection.

187

### 188 **RT53 targets metabolically active cells**

189 Although AAC11-derived peptides preferentially targeted differentiated T cells, the effect was not  
190 absolute and not all Em and Tm cells were depleted after RT53 treatment. On the other hand we  
191 observed some extent of peptide-induced cell death in all CD4+ T cell subsets, including naïve cells.  
192 This suggested that AAC11-derived peptides targeted cells with specific characteristics that were  
193 strongly enriched, but not exclusively found, within more differentiated subsets. AAC11 was  
194 previously shown to increase the survival of cancer cells during metabolic stress. Cancer cells rely on  
195 high level of glycolysis for adequate supply of biosynthetic intermediates for their life cycle. We  
196 found that the gene expression level of AAC11 was strongly correlated with the expression of  
197 numerous genes associated with cell metabolism (Fig 1B). We thus analyzed if the differential  
198 sensitivity of CD4+ T cells to RT53 treatment was related to differences in cellular metabolism.  
199 Following treatment of CD4+ T cells with RT53 but not RK16, we observed disappearance of larger  
200 CD4+ T cells (determined by FCS in flow cytometry) (Fig S3) and a decrease in the proportion of  
201 CD25<sup>high</sup> and HLA-DR<sup>high</sup> cells (Fig 6A). We next analyzed the metabolic activity [glycolysis (measured  
202 by extracellular acidification rate (ECAR) and oxidative phosphorylation (OXPHOS) (measured by  
203 oxygen consumption rate (OCR))] of living CD4+ T cells in non-treated or cells treated with RT53/RK16  
204 peptides (Fig 6B). We found that the cells that remained after RT53 treatment had lower  
205 mitochondrial respiration (basal and spare capacity) and glycolysis (basal ECAR and glycolytic reserve  
206 (ECAR after FCCP)) than cells that had not been treated or treated with RK16. We thus concluded  
207 that CD4+ T cells with the highest metabolic activity were more sensitive to the action of AAC11-  
208 derived peptides.

209

210 **RT53-surviving cells are non-permissive to HIV-1 replication**

211 To further confirm that RT53 was killing HIV-1 susceptible target cells and selecting for cells resistant  
212 to infection, we treated activated CD4+ T cells with RT53 or RK16 for 24 hours, sorted live cells from  
213 dead cells and infected sorted live cells (Fig 7A). We found that the cells that survived RT53  
214 treatment were resistant to infection with both HIV-1 Bal and VSVG-pseudotyped HIV-1 particles (Fig  
215 7A), although the inhibition was more marked for the replication competent virus. Importantly, we  
216 did not see any further increase in cell death in sorted living cells. We next sought to determine  
217 which stage of viral infection was blocked in cells surviving RT53 treatment. RT53 induced a slight  
218 decrease in the proportion of CCR5<sup>high</sup> cells (Fig S4), although this difference did not appear sufficient  
219 to explain the level of HIV inhibition found. We next measured the levels of total (as a measure of  
220 reverse transcription) and integrated viral DNA at various time points post-infection in RT53/RK16  
221 treated or untreated cells (Fig. 7B). We could detect viral DNA at 4 hours post-infection in all  
222 treatment conditions suggesting that the virus is able to enter RT53 treated cells. Levels of both total  
223 and integrated HIV-1 DNA drastically increased as a function of time in RK16-treated and untreated  
224 cells but not in RT53-treated cells. These results indicate that although the virus is able to enter live  
225 cells after RT53 treatment, these cells are non-permissive to viral replication. Thus, RT53 treatment  
226 is killing virally susceptible targets while allowing the survival of cells where HIV-1 cannot complete  
227 the first post-entry steps of its replication cycle.

228

229

230

231 **DISCUSSION**

232 In this study we showed that peptides antagonizing the antiapoptotic action of AAC11 inhibit HIV-1  
233 infection of primary CD4+ T cells. In particular, we found that RT53 was able to block infection with a  
234 large panel of lab adapted and primary viral strains by inducing apoptosis of CD4+ T cells that offered  
235 the best conditions to sustain HIV replication. Our results also suggest that the AAC11 survival  
236 pathway is involved in the regulation of T cell differentiation by facilitating the survival of subsets of  
237 cells with high metabolic activity.

238

239 We found that gene expression of the apoptosis inhibitory protein AAC11 increased with  
240 differentiation of memory CD4+ T cells. Furthermore we found a strong correlation between the  
241 expression of AAC11 and multiple genes involved in the regulation of cell metabolism. Upon  
242 antigenic stimulation CD4+ T cells upregulate their metabolic activities, in particular glucose  
243 metabolism, to cope with the bioenergetics demands necessary to exert their functions. Metabolic  
244 arrangements are also necessary to sustain long-term memory cells. Cellular metabolism and  
245 apoptosis are deeply entangled. External signals provided by TCR activation or growth factors such  
246 as IL-2 and IL-7 promote an anti-apoptotic state of the cell by increasing the levels of metabolite  
247 transporters (e.g. Glut1) that ensure the supply of nutrients necessary to sustain the bioenergetic  
248 demands of the cell. The absence of these signals provokes a limitation in the influx of nutrients that  
249 results in metabolic stress, the activation of apoptotic pathways and cell death. We show that RT53  
250 antagonized the anti-apoptotic activity of AAC11 and induced death in CD4+ T cells, and that  
251 sensitivity to RT53 increased with T cell differentiation and metabolic activity of the cells. Our results  
252 suggest that the AAC11 survival pathway may be involved in regulation of T cell immunity, a function  
253 that has not been previously described and deserves further detailed exploration.

254

255 Although the antiapoptotic action of AAC11 is well documented, the molecular mechanisms  
256 underlying its activity are still unclear. AAC11 possesses several domains predicted to be responsible

257 for protein-protein interactions (Han et al., 2012), and its expression may interfere with different  
258 mechanisms of cell death. The peptides that we tested here were designed to mimic the heptad  
259 leucine repeat region of AAC11 and to be used as competitive inhibitors that abrogate the interaction  
260 of ACC11 with some of its partners. Along these lines RT53 has been shown to exert its function  
261 without directly affecting AAC11 itself (Jagot-Lacoussiere et al., 2016). We found that AAC11  
262 expression, both on the mRNA and protein level, was unchanged with RT53 treatment (Fig S5). This  
263 indicates that de-repression of pro-apoptotic AAC11 partner(s) rather than changes in AAC11  
264 expression itself is responsible for cell death differences observed in our cells upon treatment with  
265 the peptides. Moreover, the relationship between the HIV-1 inhibition and AAC11 may not be direct  
266 but rather involves a molecular pathway with complex interplay of signaling factors the relative  
267 contribution of which to HIV-1 infection is worth investigating further. We found that among the  
268 peptides tested, those spanning the whole LZ domain were more potent inducers of cell death and  
269 blocked HIV infection more efficiently. Additionally, the fast kinetics of their activity points to an action  
270 through rearrangement of pre-existing cellular factors. In tumor cells physical interaction between the  
271 LZ domain of AAC11 and Acinus prevents Acinus pro-apoptotic cleavage by caspase 3. In our study we  
272 did not observe significant changes in the expression of Acinus upon treatment with AAC11-derived  
273 peptides (not shown) and caspase 3 activity was not significantly detected in our experimental  
274 conditions, suggesting that this pathway was not the major contributor to T cell death observed in our  
275 experiments. In contrast we found that the treatment with RT53 induced a strong increase in caspase  
276 2 activity. AAC11 has been recently shown to physically bind to the caspase recruitment domain of  
277 caspase 2 preventing its activation (Imre et al., 2017). Our results suggest that RT53 may impair this  
278 interaction and facilitate caspase 2 dimerization and initiation of apoptosis. However, RT53-induced  
279 caspase 2 activation did not prompt cell death in all cells suggesting that additional factors intervened  
280 downstream in this process and were not equally expressed by all cells.

281

282 The main goal of this study was to explore if interfering with AAC11 activity could affect HIV infection.  
283 We show that AAC11 peptides, particularly RT53, strongly impaired infection with different HIV-1  
284 strains and SIV<sub>mac251</sub>. There was a close association between peptide-induced cell death and inhibition  
285 of HIV infection. Not only the rates of cell death induced by AAC11 peptides correlated with the extent  
286 of HIV inhibition, but precluding apoptosis by increasing extracellular levels of K<sup>+</sup> also preserved the  
287 infectability of CD4<sup>+</sup> T cells. Moreover, pre-treatment of CD4<sup>+</sup> T cells with RT53 selected for cells that  
288 were strongly resistant to HIV-1 infection (for instance reverse transcription was severely impaired in  
289 these cells). This and the fact that RT53 depleted highly metabolic cells are in agreement with the  
290 observation that a rich metabolic environment is necessary for HIV replication (Valle-Casuso,  
291 submitted). It is interesting to notice that AAC11 gene expression was correlated with the expression  
292 of RRM2, an enzyme that is critical for the de novo synthesis of dNTPs, and the depletion of which  
293 blocks HIV-1 infection in macrophages and dendritic cells (Allouch et al., 2013; Valle-Casuso et al.,  
294 2017). AAC11 mRNA levels also correlated with the expression of other genes that have been  
295 associated with the HIV replication cycle in particular trafficking (i.e. CFL1, DYNC1H1, ACTB) and  
296 transcription (i.e. CDK9, NFKB1) suggesting that additional steps of the HIV-1 cycle may be impaired  
297 in cells resisting RT53-induced cell death. Of note, although we observed a very consistent inhibition  
298 of infection with VSVG-pseudotyped NL4.3 single cycle particles upon treatment with AAC11 peptides,  
299 the inhibition was more important when we used wild type viruses, either R5 or X4. There are several  
300 possible explanations to this. First, the additional block in HIV infection observed when we used WT  
301 viruses could be due to a cumulative effect of multiple infection cycles. Second, cells selected upon  
302 treatment with AAC11 may offer an extra restriction to WT virus. For instance cells expressing high  
303 levels of CCR5 were depleted upon treatment with RT53. In contrast RT53 sharply increased surface  
304 levels of phosphatidylserine, which may facilitate entry mediated by VSVG (Carneiro et al., 2002). We  
305 cannot exclude that treatment with AAC11-derived peptides not only depleted the CD4<sup>+</sup> T cells that  
306 were most susceptible to HIV-1 infection but also induced viral restriction in the cells that survived.  
307 For instance, AAC11 has been shown to regulate the cell cycle, and interference with AAC11 activity



308 might affect G1 into S transition (Navarro et al., 2013) and HIV infection (Wang et al., 2011)(Clark et  
309 al., 2000).

310

311 Our results show that AAC11 antiapoptotic pathway is active in CD4+ T cells that are particularly  
312 susceptible to HIV-1 infection. It remains to be established if this pathway may contribute to  
313 persistence of infected cells on the long-term. The mechanisms by which an infected cell survives and  
314 persists over time are not completely clear, as HIV has cytopathic effects that lead to the death of  
315 most infected CD4+ T cells. It has been recently reported that persistent latently infected CD4+ T cells  
316 are intrinsically resistant to killing by HIV-specific CD8+ T cells (Huang et al., 2018), which may indicate  
317 that the persistent reservoir may be seeded in cells programmed to resist cell death. Like the  
318 mechanisms associated with HIV persistence, it is also unclear how some T cells survive to become  
319 long-lived memory cells. While most T cells undergo apoptosis at the end of the immune response  
320 when environmental signals wane, a few cells survive even in the absence of growth factors (Sallusto  
321 et al., 2010). AAC11 prevents apoptosis of tumor cells in conditions of nutrient deprivation and in the  
322 absence of growth factors. We can therefore speculate that the AAC11 survival pathway might play a  
323 similar role in promoting persistence of memory CD4+ T cells and HIV infection.

324

325 Although the differences between infection with WT viruses and single-cycle VSVG pseudotyped  
326 particles were visible both when cells were treated with RT53 hours before infection or at the time of  
327 infection, we cannot exclude that infection with WT HIV may induce cellular changes that can further  
328 increased the susceptibility of infected cells to the action of AAC11-derived peptides. HIV has been  
329 shown to induce metabolic changes in infected cells and in particular an increase in the expression of  
330 Glut1-3 (Sorbara et al., 1996). It has been recently shown that BIRC5 (a.k.a. Api4 or survivin), a  
331 member of the inhibitor of apoptosis protein family, is upregulated in CD4+ T cells during HIV-1  
332 infection and contributes to the persistence of infected cells (Kuo et al., 2018). Upregulation of BIRC5  
333 on infected CD4+ T cells was triggered by OX40, a costimulatory receptor that promotes T cell

334 differentiation and survival (Huddleston et al., 2006; Redmond et al., 2009) and contributes to the  
335 metabolic boost necessary for T cell activation (Pacella et al., 2018). It is thus possible that HIV-1  
336 exploits/activates several cellular survival programs associated with T cell activation and metabolic  
337 activity to ensure its persistence.

338

339

340 **MATERIAL AND METHODS**

341 **Peptides, antibodies and probes**

342 Peptides (Proteogenix) were received as dry powder and reconstituted with water for use. Peptide  
343 sequences are as follows.

344 RT53: RQIKIWFQNRRMKWKKAKLNAEKLKDFKIRLQYFARGLQVYIRQLRLALQGKT,

345 RT39: RQIKIWFQNRRMKWKKLQYFARGLQVYIRQLRLALQGKT,

346 RL29: RQIKIWFQNRRMKWKKYFARGLQVYIRQL,

347 RQ26: RQIKIWFQNRRMKWKKLQYFARGLLQ,

348 RK16: RQIKIWFQNRRMKWKK.

349 Antibodies and dyes used for flow cytometry and FACS sorting are as follows: LIVE/DEAD™ Violet  
350 Viability dye (ThermoFisher), CD3-PE (clone SK7, Biolegend), CD4-A700 (clone OKT4, eBioscience),  
351 CD45RA-APC-Cy7 (clone HI100, Biolegend), CCR7-Pe-Cy7 (clone GO43H7, Biolegend), CD27-APC (clone  
352 M-T271, Myltenyi), CD25-PE-Dazzle594 (clone M-A251, Biolegend), HLA-DR-PerCP-Cy5.5 (clone G46-  
353 6, Biolegend), CCR5-PE (clone 3A9, BD), p24-FITC (clone KC57, Coulter). Caspase activity was assayed  
354 with the following probes according to the manufacturer's instructions: caspase 1 660-YVAD-FMK  
355 probe (FLICA 660 Caspase-1 Assay Kit, ImmunoChemistry Technologies<sup>LLC</sup>), caspase 2 FAM-VDVAD-  
356 FMK probe (FAM-FLICA Caspase-2 Assay Kit, ImmunoChemistry Technologies<sup>LLC</sup>), caspase 3/7 SR-  
357 DEVD-FMK probe (SR-FLICA® Caspase 3/7 Assay Kit, ImmunoChemistry Technologies<sup>LLC</sup>). FACS sorting  
358 was performed on BD Aria and flow cytometry acquisition on BD LSRII.

359

360 **Isolation and culture of primary human CD4+ T cells**

361 Healthy donor blood prepared as a buffy coat was obtained from *Etablissement Français du Sang (EFS)*  
362 (agreement with Institut Pasteur C CPSL UNT, 15/EFS/023). Blood was overlaid on Ficol (EuroBio) at  
363 a ratio of 2:1 v/v blood to Ficol and centrifuged at 1,800 rpm for 30 minutes at a minimum  
364 acceleration/deceleration to obtain peripheral blood mononuclear cells (PBMCs). CD4+ T cells were  
365 then purified from PBMCs by negative selection using StemCell EasySep™ Human CD4+ T cell Isolation

366 Kit. Cells were counted and cultured in RPMI-1640 containing Glutamax (ThermoFisher), 10% fetal  
367 bovine serum (FBS), penicillin-streptomycin (ThermoFisher) (100 U/ml) and IL-2 (Myltenyi) (100U/ml)  
368 (thereafter referred to as culture medium) at 10<sup>6</sup> cells/ml in 37° degree, 5% CO<sub>2</sub> humidified incubator.  
369 Cells were suboptimally activated with soluble anti-CD3 (clone UCHT-1) (Biolegend) for 5 days prior to  
370 infection or analysis as previously described (Sáez-Ciri6n et al., 2011).

371

### 372 **Isolation and culture of primary infected macaque CD4+ T cells**

373 Macaque splenic CD4+ T cells were obtained from Cynomologus macaques (*Macaca fascicularis*) that  
374 were imported from Mauritius island and housed at *Commissariat à l'Energie Atomique et aux*  
375 *Energies Alternatives (CEA)* at Fontenay-aux-Roses in France under the compliance with the Standards  
376 for Human Care and Use of Laboratory Animals (Assurance number A5826-01). These animals were  
377 part of the pVISCONTI study, which received ethics approval under the number APAFIS#2453-  
378 2015102713323361 v2. They were infected intravenously with 1000 AID<sub>50</sub> of SIV<sub>mac251</sub> and sacrificed  
379 at a study end point to obtain spleen samples. Splenic macaque CD4+ T cells were purified by  
380 mechanical disruption of a spleen sample in RPMI media using GengleMACS™ dissociator (Miltenyi)  
381 followed by cell overlay over Ficol (EuroBio) (diluted with PBS to 90% prior to use) and centrifugation  
382 at 350g for 20min. Cells were then subjected to red cell lysis and then to CD4+ T cell negative selection  
383 using CD4+ T cell negative selection kit (Myltenyi). Cells were cultured in the culture media overnight  
384 prior to incubation with peptides. Viral spread from in vivo infected cells was monitored by ELISA  
385 quantification of p27 (Expressbio) levels on culture supernatants.

386

387

### 388 **Infection and peptide treatment of primary human CD4+ T cells**

389 Activated CD4+ T cells were infected with either: Bal (2.9 ng/ml p24), BX08 (21 ng/ml p24), DH12 (3  
390 ng/ml p24), 132w (6.3 ng/ml p24), NL4.3 (7 ng/ml p24), vesicular stomatitis virus glycoprotein  
391 (VSVG) pseudotyped-Δenv-Δnef-GFP (7 ng/ml p24) or SIV<sub>mac251</sub> (36.7 ng/ml p27) viruses by

392 centrifuging at 1,200g for 1 hour at room temperature and then incubating for 1 hour at 37° degrees  
393 in a humidified 5% CO<sub>2</sub> incubator (spinoculation in text). Cells were then washed once with PBS,  
394 incubated in the culture medium and treated with peptides at 6 μM concentration unless otherwise  
395 indicated. Cell death and infection were measured on day 3 post-infection unless otherwise stated.  
396 Cell death was evaluated using flow cytometry (LIVE/DEAD™ Violet Viability dye (ThermoFisher))  
397 and infection was evaluated by either flow cytometry (intracellular p24 staining) or p24/p27 ELISA  
398 (XpressBio).

399

#### 400 **Real-time flow cytometry**

401 Cells were washed once with PBS and incubated in Annexin buffer (10 mM HEPES, 140 mM NaCl, 2.5  
402 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  
403 for 15 minutes. 7-AAD (Biolegend) was added for the last 5 minutes of incubation. Cells were then  
404 directly passed through the flow cytometer to acquire fluorescence vs time without washing.  
405 Alternatively, cells were stained with asanate potassium green 2 (APG-2) AM (Abcam), a fluorescent  
406 K<sup>+</sup> indicator, at a final concentration of 1 μM in plain RPMI-1640 medium for 30 minutes at room  
407 temperature, washed twice with plain RPMI-1640, resuspended in PBS at 10<sup>6</sup> cells/ml and incubated  
408 with 7-AAD (Biolegend) for 5 minutes before acquisition. 6 μM peptides were added after 2 minutes  
409 of baseline acquisition and acquisition continued for additional 28 minutes.

410

#### 411 **Measurements of cellular metabolism**

412 Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured on  
413 Seahorse XF96 analyzer using Seahorse XF Cell Mito Stress Test Kit (Agilent). Briefly, activated CD4+ T  
414 cells were incubated with 6 μM RT53 or RK16 for 4 hours at 37°C. Cells were then counted, washed  
415 in a Seahorse XF medium (Agilent Seahorse XF base medium with 2mM Glutamax (Agilent, 102365-  
416 100) containing 10mM glucose (Sigma), 2mM sodium pyruvate (LifeTechnologies) and adjusted to  
417 pH 7.4). Equal number of live cells was seeded at 2\*10<sup>5</sup> cells per well in XF96 V3 PS plates (Seahorse

418 Bioscience) precoated with 0.5 mg/ml Corning® Cell-Tack™ Cell and Tissue Adhesive (Corning,  
419 354240), and incubated for a minimum of 30 minutes in a CO<sub>2</sub>-free, 37°C incubator prior to  
420 acquisition. The following drugs were placed at injection ports: Seahorse XF medium (port A), 2.5  
421 μM Oligomycin (port B), 0.9 μM FCCP (port C), 1 μM rotenone and 1 μM antimycin A (port D).

422

### 423 **Quantitative RT-PCR**

#### 424 *Evaluating the expression of AAC11 and other genes in CD4+ T cell memory subsets*

425 The expression levels of AAC11 and other genes on CD4+ T cell subsets were analyzed in a previous  
426 study (Valle-Casuso, submitted). Briefly, the total RNA from 5\*10<sup>4</sup> cells was extracted using RNA  
427 Trace Kit (Macherey Nagel), treated with DNase, reverse transcribed using Reverse Transcription  
428 Master Mix (Fluidigm), pre-amplified using PreAmp Master Mix (Fluidigm) and treated with  
429 exonuclease I (New England Biolabs). Samples were then mixed with SsoFast EvaGreen Supermix  
430 with Low ROX (Biorad), DNA Binding Dye (Fluidigm), and assay mix (assay loading reagent (Fluidigm)  
431 and Delta Gene primers (Fluidigm)). The expression levels were read on Biomark HQ system  
432 (Fluidigm). The expression levels of BECN1 were used for normalization. Gene expression values are  
433 plotted as 2<sup>-ΔΔCt</sup>.

434

#### 435 *Evaluating the expression of viral gene products*

436 Cells were collected by centrifugation and dry pellet was stored at -80°C until DNA extraction. DNA  
437 was extracted using NucleoSpin® Tissue Kit (Macherey Nagel). Real time PCR (RT-PCR) to quantify  
438 total and integrated HIV-1 DNA was performed as described previously (David et al., 2006) using  
439 TaqMan™ Universal PCR Master Mix (ThermoFisher). Briefly, total viral DNA was quantified using  
440 the following primers and probe: CTT TCG CTT TCA AGT CCC TGT T (forward), AGA TCC CTC AGA CCC  
441 TTT TAG TCA (reverse), (FAM)-TGG AAA ATC TCT AGC AGT GGC GCC C-(BHQ1) (probe). 8E5 cell line  
442 containing a single viral copy per cell was used as a standard. Integrated viral DNA was quantified by  
443 first pre-amplifying the DNA using AccuTaq™ LA DNA Polymerase (Sigma) using the following

444 primers: AGC CTC CCG AGT AGC TGG GA (FirstAluF); TTA CAG GCA TGA GCC ACC G (FirstAluR); CAA  
445 TAT CAT ACG CCG AGA GTG CGC GCT TCA GCA AG (NY1R). Second DNA amplification round was  
446 performed with TaqMan™ Universal PCR Master Mix (ThermoFisher) using the following primers:  
447 AAT AAA GCT TGC CTT GAG TGC TC (NY2F); CAA TAT CAT ACG CCG AGA GTG C (NY2R); FAM-AGT  
448 GTG TGC CCG TCT GTT GTG TGA CTC-TAMRA (NY2ALU probe). HeLa cells line containing HIV-1  
449 integrated DNA was used as a standard (David et al., 2006). Results were normalized to ng actin  
450 using Human DNA standard (Sigma). Primers used for quantification of actin were: TGC ATG AGA  
451 AAA CGC CAG TAA (forward); ATG GTC GCC TGT TCA CCA A (reverse); (FAM)-TGA CAG AGT CAC CAA  
452 ATG CTG CAC AGA A-(TAMRA) (probe).

453

#### 454 **Statistical analysis**

455 Statistical analysis was performed using GraphPad Prism software. Linked parametric or non-  
456 parametric one-way ANOVA or two-way ANOVA were used depending on the experiment. \* denotes  
457  $p \leq 0.05$ , \*\* denotes  $p \leq 0.01$ , \*\*\* denotes  $p \leq 0.001$ , \*\*\*\* denotes  $p \leq 0.0001$ . Dunnett's, Dunn's or  
458 Holm-Sidak's multiple comparisons tests were used for post-hoc analysis.

459

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465

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471

472 **Author Contributions**

473 AM, AD, JCV-C, VM performed experiments; AM, AD, JCV-C, VM, SV and AS-C analyzed the data; JLP  
474 and CP provided key reagents; JLP and AS-C conceived the study; AM, AD, JCV-C and AS-C designed  
475 the experiments; AS-C supervised the study; AM and AS-C drafted the article; and all authors critically  
476 reviewed the manuscript.

477



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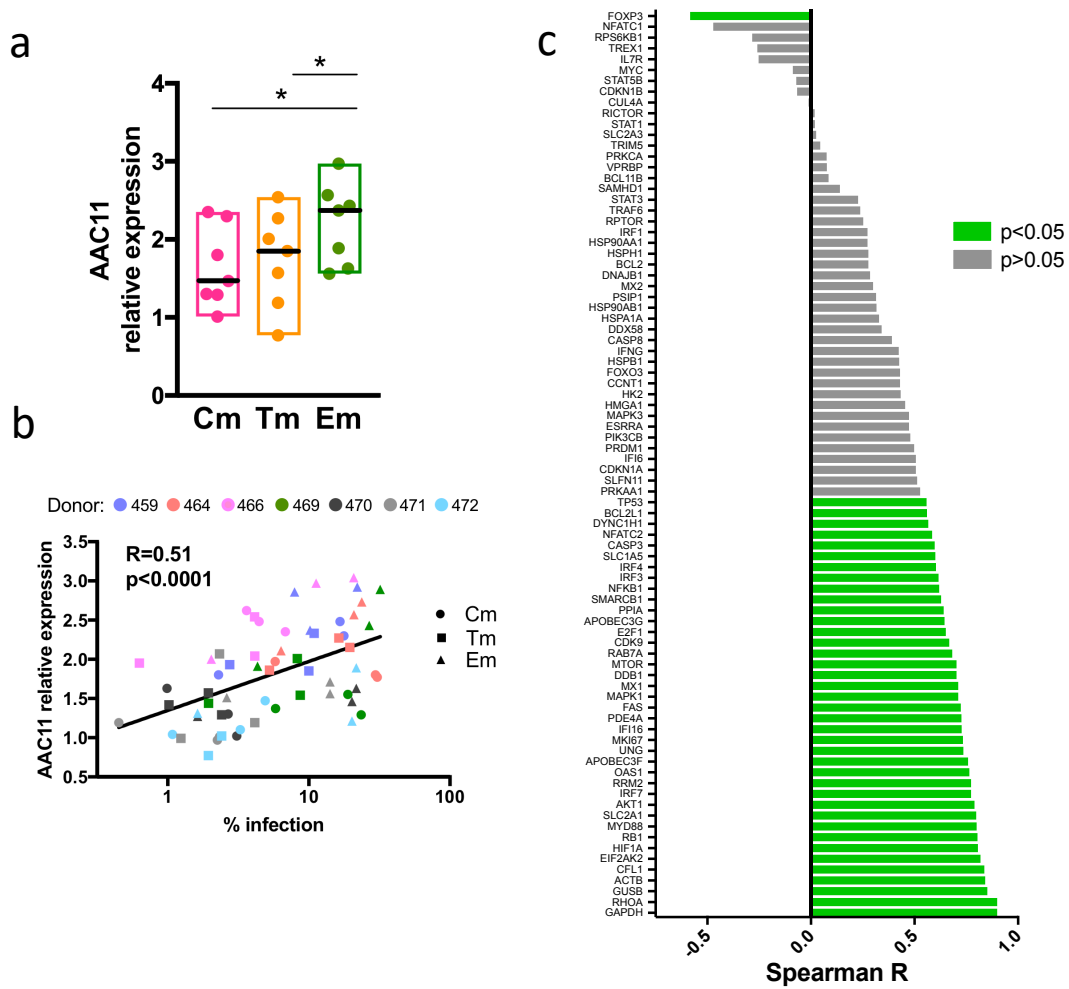
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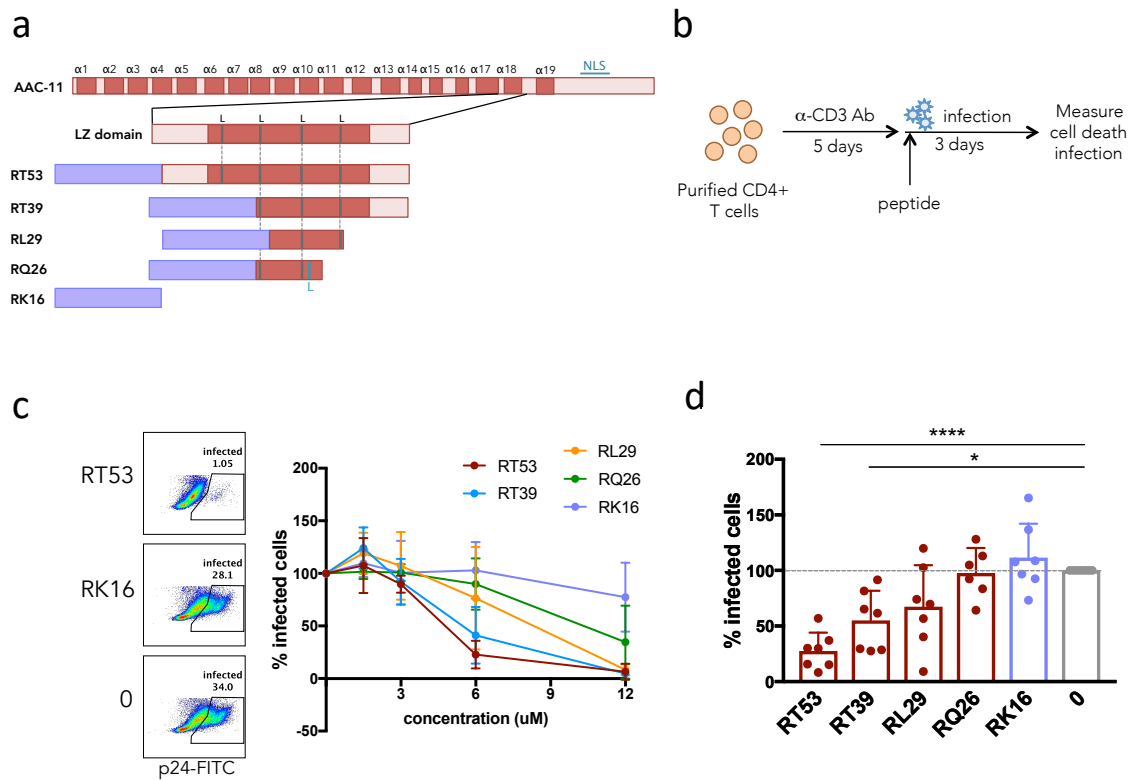
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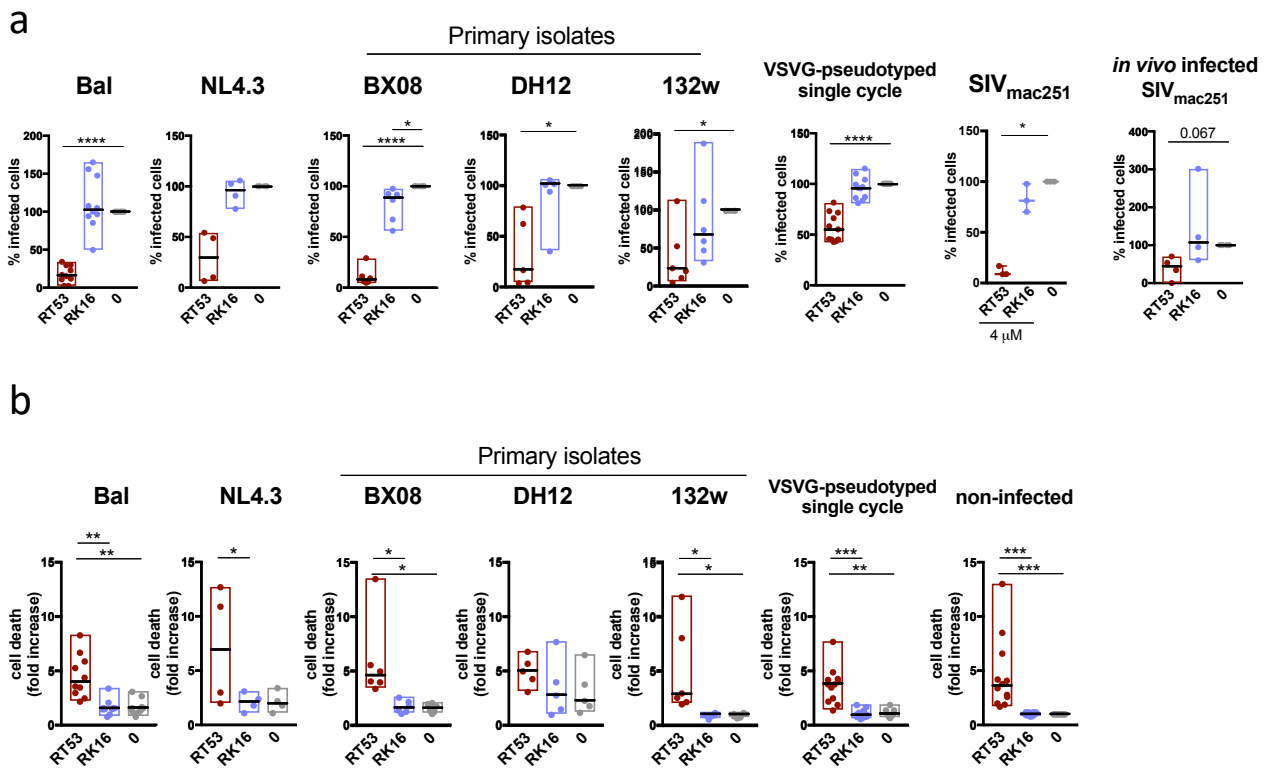
FIGURES AND FIGURE LEGENDS



**Figure 1. AAC11 gene expression correlates with the proportion of infection in each memory CD4+ T cell subset and is associated with expression of various cell cycle and metabolism genes. A.** Suboptimally activated CD4+ T cell memory subsets (Cm, Tm, Em) were analyzed for the expression of AAC11 at the time of infection. **B.** Suboptimally activated (day 3 and 5) or non-activated Cm, Tm and Em subsets were analyzed for the levels of AAC11 gene expression and correlated with % of infected cells in each subset at day 3 post-infection **C.** The correlation of AAC11 gene expression with other genes in a 96-gene array.



**Figure 2. AAC11-derived peptides inhibit HIV-1 infection.** **A.** Schematic representation of AAC11-derived peptides indicating LZ domain **B.** Experimental scheme of treatment of infected cells with AAC11-derived peptides **C.** Representative flow cytometry plot of p24 staining in live, untreated or cell treated with 6  $\mu\text{M}$  RT53 or RK16. A dose-response curve of the effect of AAC11-derived peptides on HIV-1 infection in living cells **D.** The proportion of infection as compared to non-treated control among living cells infected with HIV-1 BaL and treated with 6  $\mu\text{M}$  of indicated peptides.

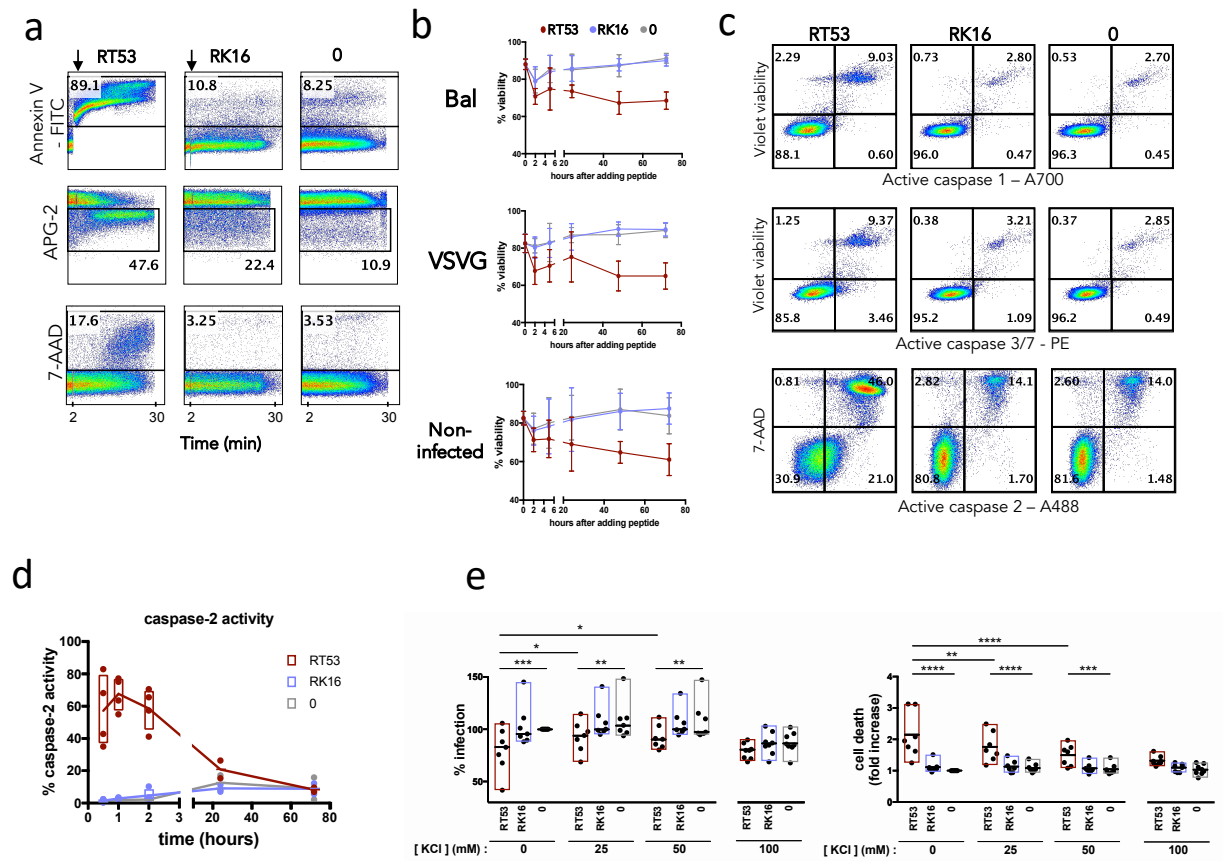


**Figure 3. RT53 inhibits infection of diverse HIV-1 strains and SIV.** Activated primary CD4<sup>+</sup> T cells were spinoculated with different HIV-1 viral strains and incubated with 6  $\mu$ M RT53 or RK16 for 3 days

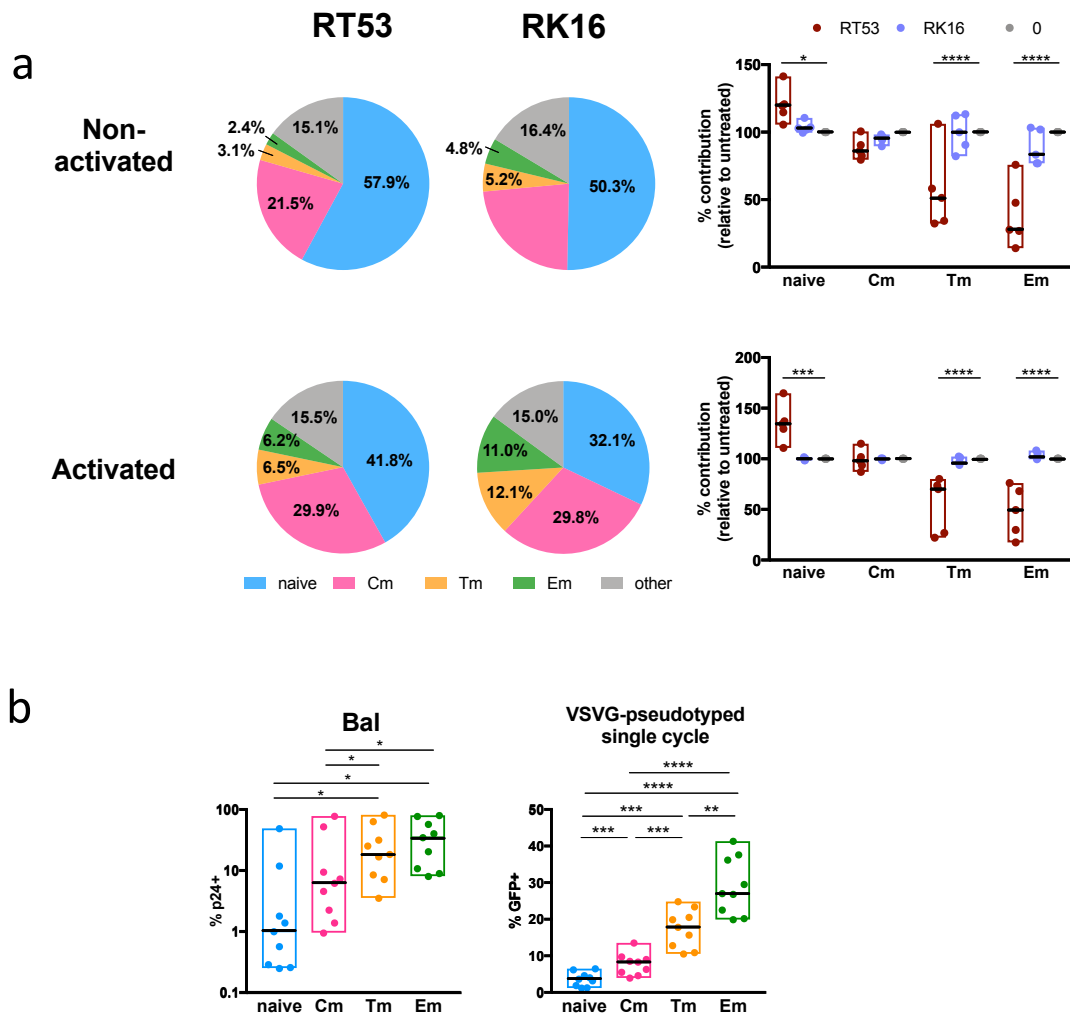
**A.** Infection was measured by flow cytometry (intracellular p24) or ELISA (p24/p27) in supernatants

**B.** Cell death was measured by flow cytometry (LIVE/DEAD<sup>TM</sup> Violet Viability dye). Values are expressed as a range (min-max) with median indicated. Each symbol represents experiments with cells from a different donor.

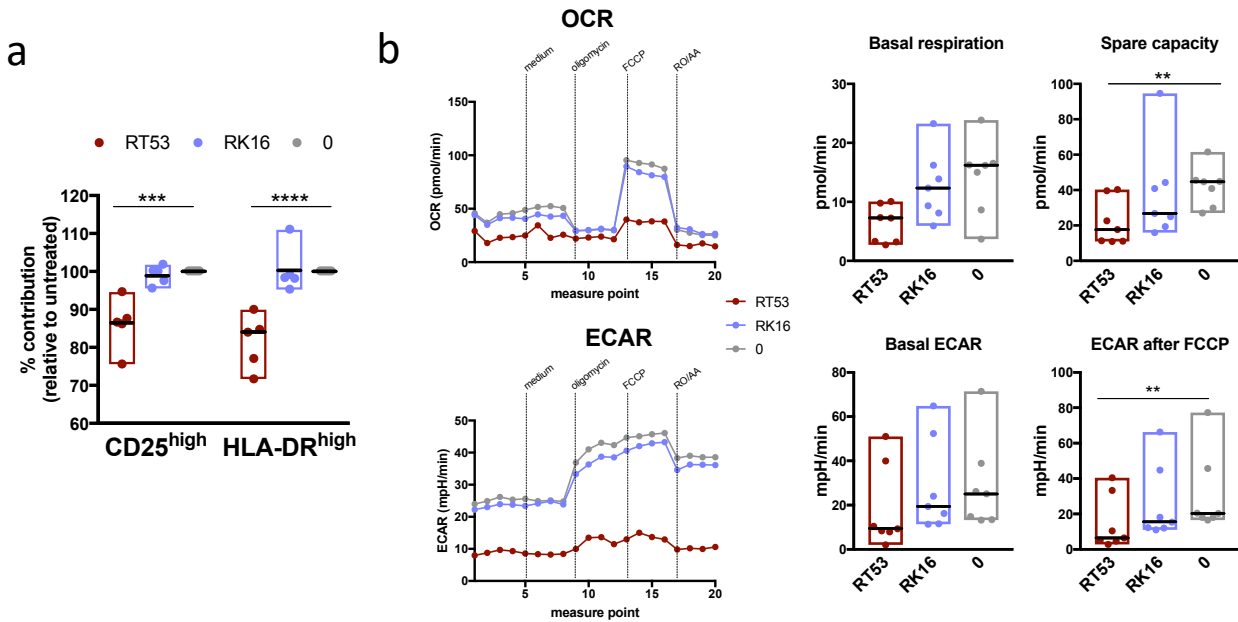




**Figure 4. RT53-mediated cell death is rapid and is associated with caspase 2 activity.** **A.** Cells were placed in Annexin V-FITC and 7-AAD staining buffer or loaded with fluorescent K<sup>+</sup> indicator (APG-2) and run on a flow cytometer to acquire fluorescence over time. Time point of peptide addition (after 2 minutes of acquisition) is indicated with an arrow **B.** Cells infected by spinoculation were incubated with 6  $\mu$ M RT53 or RK16 and viability determined by trypan blue **C.** Cells were incubated with 6  $\mu$ M RT53 or RK16 for 2 hours and stained with probes for active caspases **D.** Peptide treated cells were stained with caspase 2 activity probe at various time points post peptide treatment **E.** Cells pre-infected 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 hours. Infection and cell death were then evaluated by flow cytometry. Symbols represent experiments with cells from different donors. Bars represent min-max range with medians indicated.

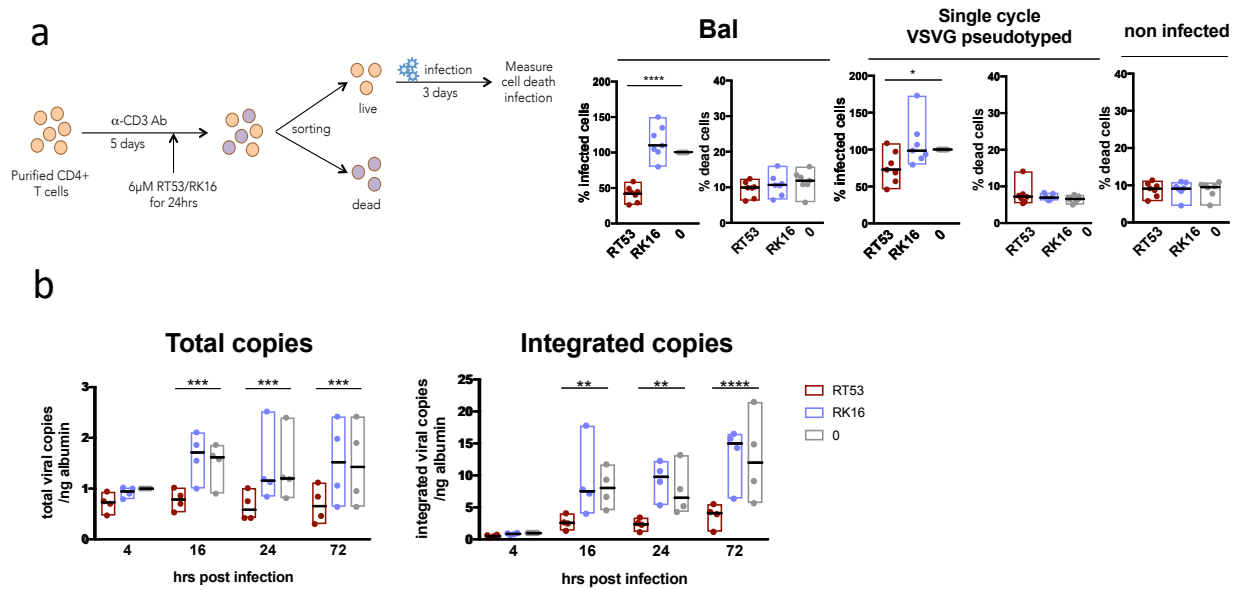


**Figure 5. RT53 preferentially targets Tm and Em cells to cell death. A.** *In vitro* activated or non-activated CD4<sup>+</sup> T cells were pulsed with 6  $\mu$ M RT53 or RK16 for 5 hours, harvested and stained for markers of CD4<sup>+</sup> T cell memory subsets. **B.** *In vitro* activated CD4<sup>+</sup> T cells were infected with HIV-1 Bal or VSVG-pseudotyped HIV-1 viral particles. On day 3 of infection, cells were phenotyped for CD4<sup>+</sup> T cell memory subpopulations, infection (intracellular p24) and cell death by flow cytometry. % of p24 or GFP<sup>+</sup> cells in each subset is indicated. Values are expressed as min-max range with median indicated.



**Figure 6. RT53 preferentially targets activated and most metabolically active cells. A. *In vitro***

activated cells were pulsed with 6  $\mu$ M RT53 or RK16 for 5 hours. Surface expression of CD25 and HLA-DR was evaluated by flow cytometry **B.** OCR and ECAR values of CD4<sup>+</sup> T cells from one representative donor (left). 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 (right). Values are expressed as min-max range. Median is indicated.



**Figure 7. RT53-surviving cells are resistant to HIV infection. A.** *In vitro* activated CD4+ T cells were incubated with 6  $\mu$ M RT53 or RK16 for 24 hours. Live cells were then sorted from dead cells using fluorescently activated cell sorting (FACS) and live cells were infected with indicated HIV-1 strains. Cell death and infection were evaluated by flow cytometry 3 days later. **B.** Total and integrated viral copies evaluated at various time points post-infection in cells infected with VSVG-pseudotyped HIV-1 viral particles and treated with 6  $\mu$ M RT53 or RK16. Values are normalized to untreated 4 hours post-infection condition and expressed as min-max range with median indicated.

## **SUPPLEMENTARY INFORMATION**

### **SUPPLEMENTARY METHODS**

#### **Immunofluorescence microscopy**

Activated CD4<sup>+</sup> T cells were incubated with 6  $\mu$ M RT53 or RK16 (Proteogenix) for 2 hours. Cells were then washed twice with PBS, immobilized on poly-lysine coated coverslips, fixed with 4% paraformaldehyde for 10 minutes at room temperature (RT), neutralized with 50mM NH<sub>4</sub>Cl for 10min at room temperature and washed twice with PBS. All antibody incubations and washes were performed in 1% bovine serum albumin (BSA) in PBS. 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 hour at RT, washed and incubated with secondary antibodies anti-mouse IgG-A488 (LifeTechnologies, A11029) and anti-rabbit IgG-A647 (LifeTechnologies, A31573) at 1/1000 dilution for 30 minutes at RT. Coverslips were then washed once in PBS and mounted using Fluoromont G (ThermoFisher) mounting medium.

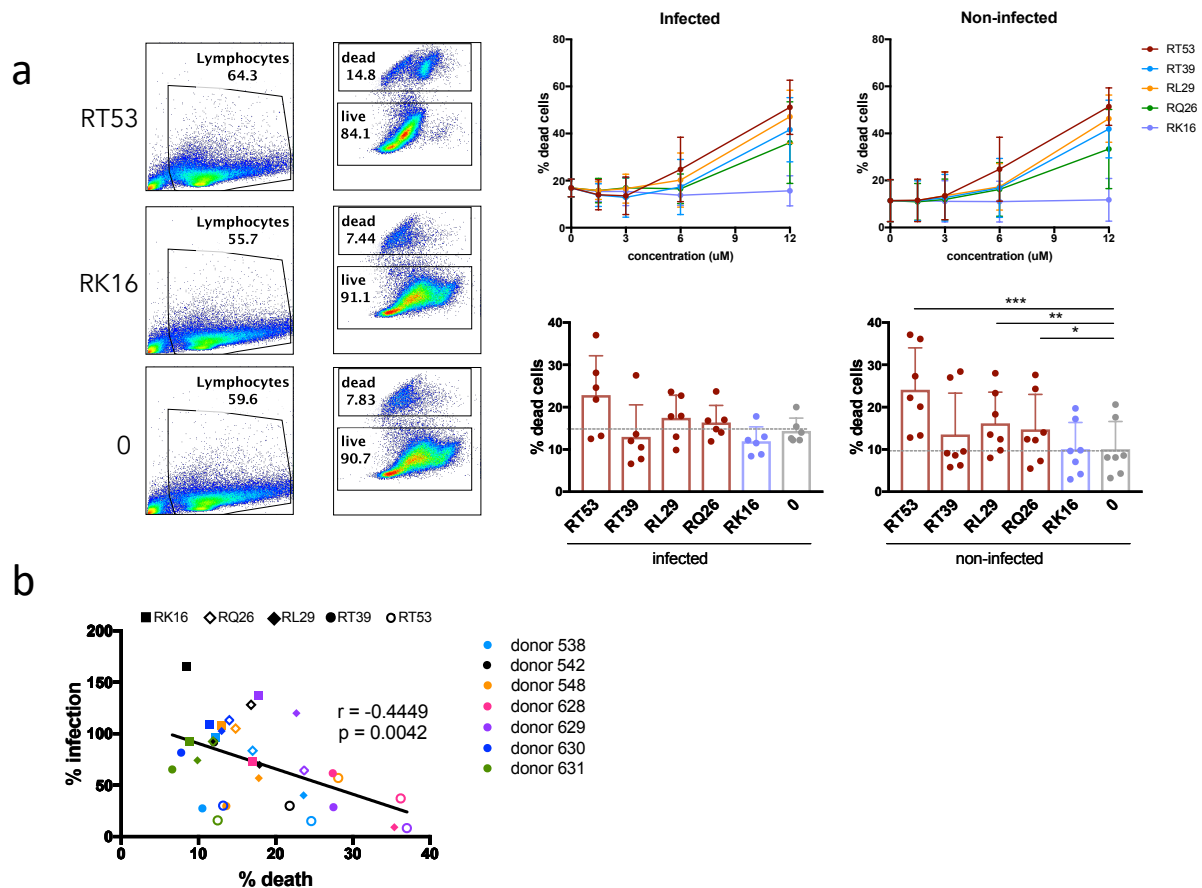
#### **AAC11 Western blot**

10<sup>6</sup> cells was pelleted and stored as dry pellet in -80°C until use. The cell pellet was lysed in a lysis buffer (150mM NaCl, 50mM pH 7.4 Tris, 1mM pH 8 EDTA, 1% Triton X-100, containing 1 tablet of complete EDTA-free protease inhibitor cocktail (Sigma) per 10ml in water) for 20min on ice with occasional vortexing. 30  $\mu$ g total protein, previously denatured in the presence of 1% v/v  $\beta$ -mercaptoethanol, was ran on a 4-15% gradient SDS-PAGE gel (Mini Protean TGX Stain-Free gel, Biorad). Samples were then transferred on PVDF membrane (Dutscher Dominique), blocked in 5% BSA in Tris-Buffered Saline 0.1% tween-20 (TBST) and blotted with anti-AAC11 Ab (abcam, ab99307) at 1/2,500 dilution overnight at 4°C in TBST, washed, and blotted by anti-rabbit HRP (Sigma, A0545) at 1/1,000 dilution in TBST for 45 minutes at RT. Membranes were also blotted with anti- $\beta$ -actin (Sigma, A5316) at 1/1,000 dilution for 45 minutes, followed by goat anti-mouse HRP (Sigma) as controls. Membrane was developed with ECL reagent (Thermo Scientific).

### **AAC11 RT-PCR**

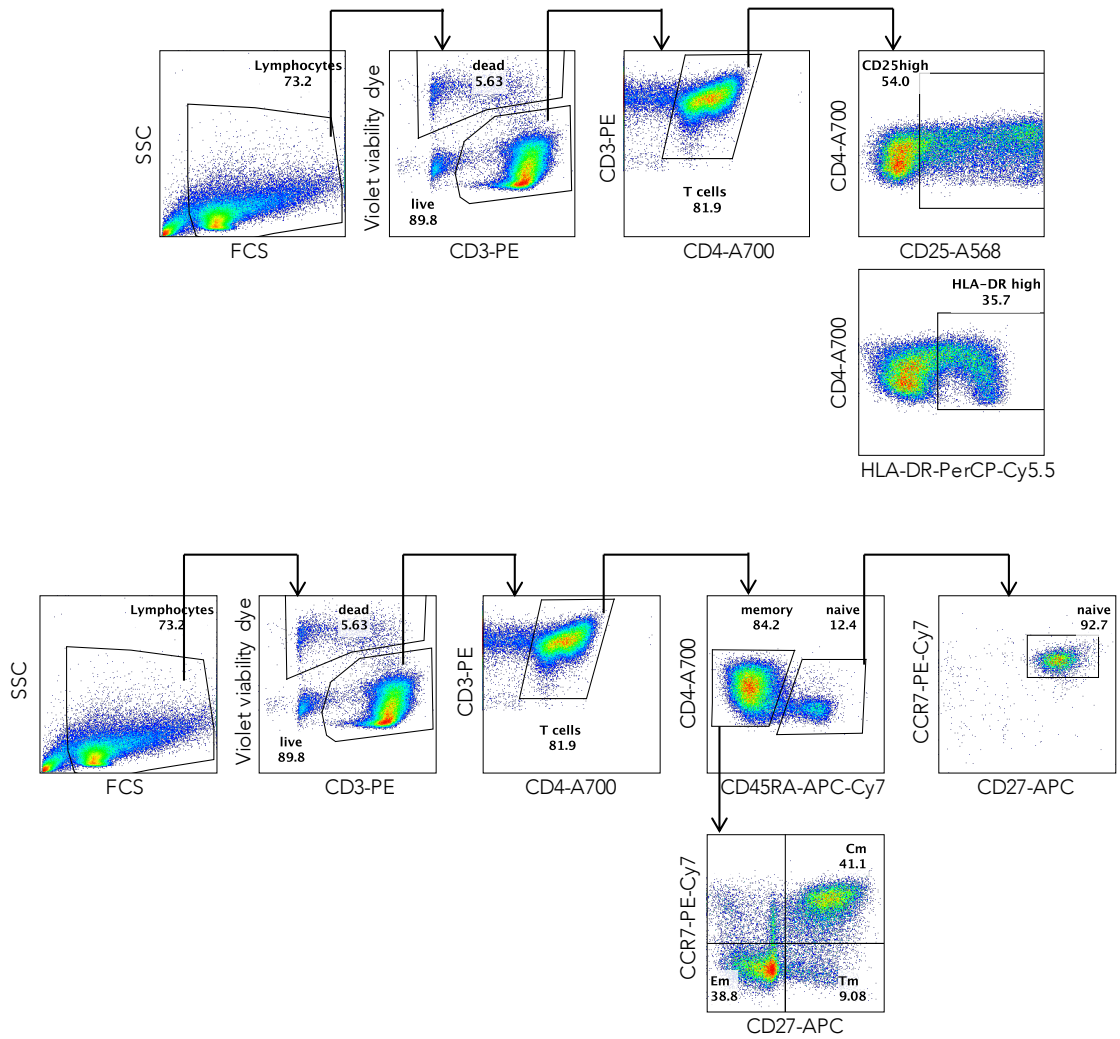
Cells were collected by centrifugation, lysed in RA1 buffer (Macherey-Nagel) and stored at -80°C until RNA extraction. RNA was purified using RNA isolation kit (Macherey-Nagel) and converted to cDNA using SuperScript II (Thermo Fisher). RT-PCR was performed on 7500 Real Time PCR System (Applied Biosystems) using TaqMan Universal Master Mix II, with UNG (Thermo Fisher) with the AAC11 (Hs00362482\_g1) and BECN1(Hs01007018\_m1) FAM-MGB-primer-probe pairs (Thermo Fisher).  $\Delta$ Ct was calculated using BECN1 and  $\Delta\Delta$ Ct was calculated using reference *ex vivo* purified CD4+ T cell sample.

## SUPPLEMENTARY FIGURES



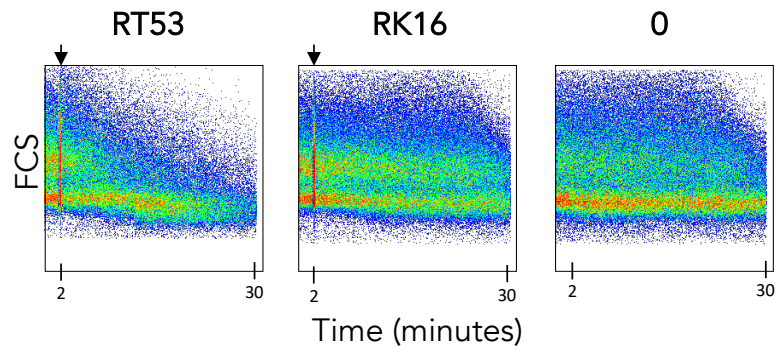
**Figure S1. Decrease in HIV-1 infection is associated with cell death**

**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 AAC11-derived peptides after spinoculation with HIV-1 Bal and cell death was evaluated by flow cytometry on day 3 of infection **B.** Proportion of infected cells among live cells vs cell death in different donors' CD4<sup>+</sup> T cells incubated with 6  $\mu$ M indicated peptides.

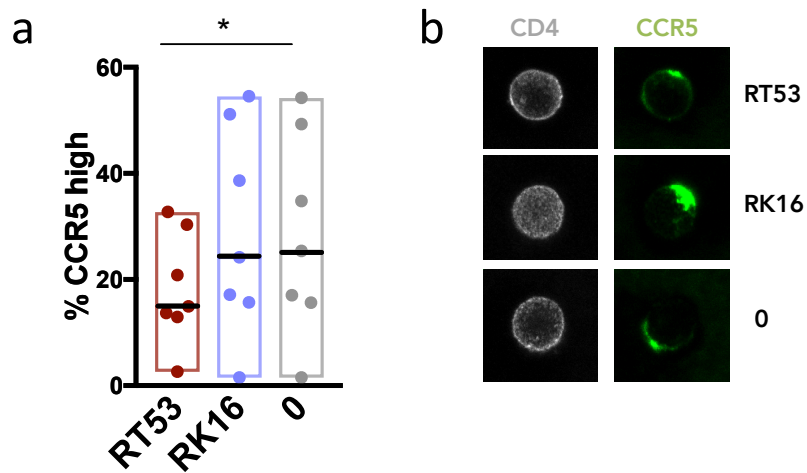


**Figure S2.** Example of the flow cytometry gating strategy for CD25 and HLA-DR activation markers as well as naïve, Cm, Tm and Em CD4<sup>+</sup> T cell subsets.

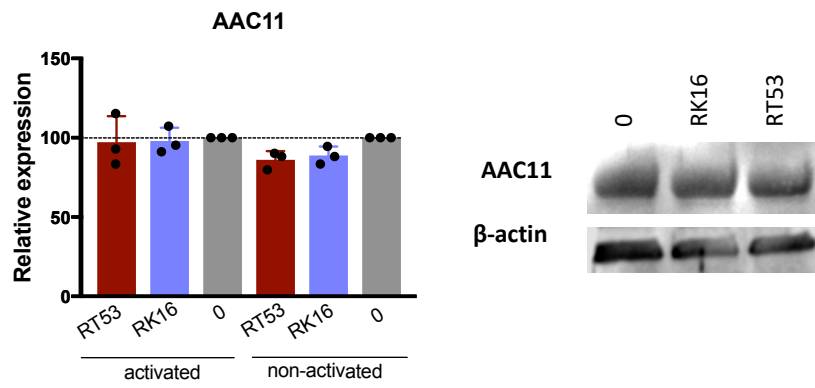




**Figure S3.** RT53 treatment preferentially targets large cells (evaluated by FSC parameter by real-time flow cytometry).



**Figure S4.** CCR5 expression and distribution upon RT53/RK16 treatment **A.** Proportion of CCR5<sup>high</sup> expressing cells and **B.** CCR5 cell surface distribution among *in vitro* activated cells after 5 hours of treatment with 6  $\mu$ M RT53 or RK16.



**Figure S5.** AAC11 gene (*in vitro* activated or non-activated CD4+ T cells) and protein expression (*in vitro* activated CD4+ cells) upon treatment with 6  $\mu$ M RT53/RK16 for 5 hours.

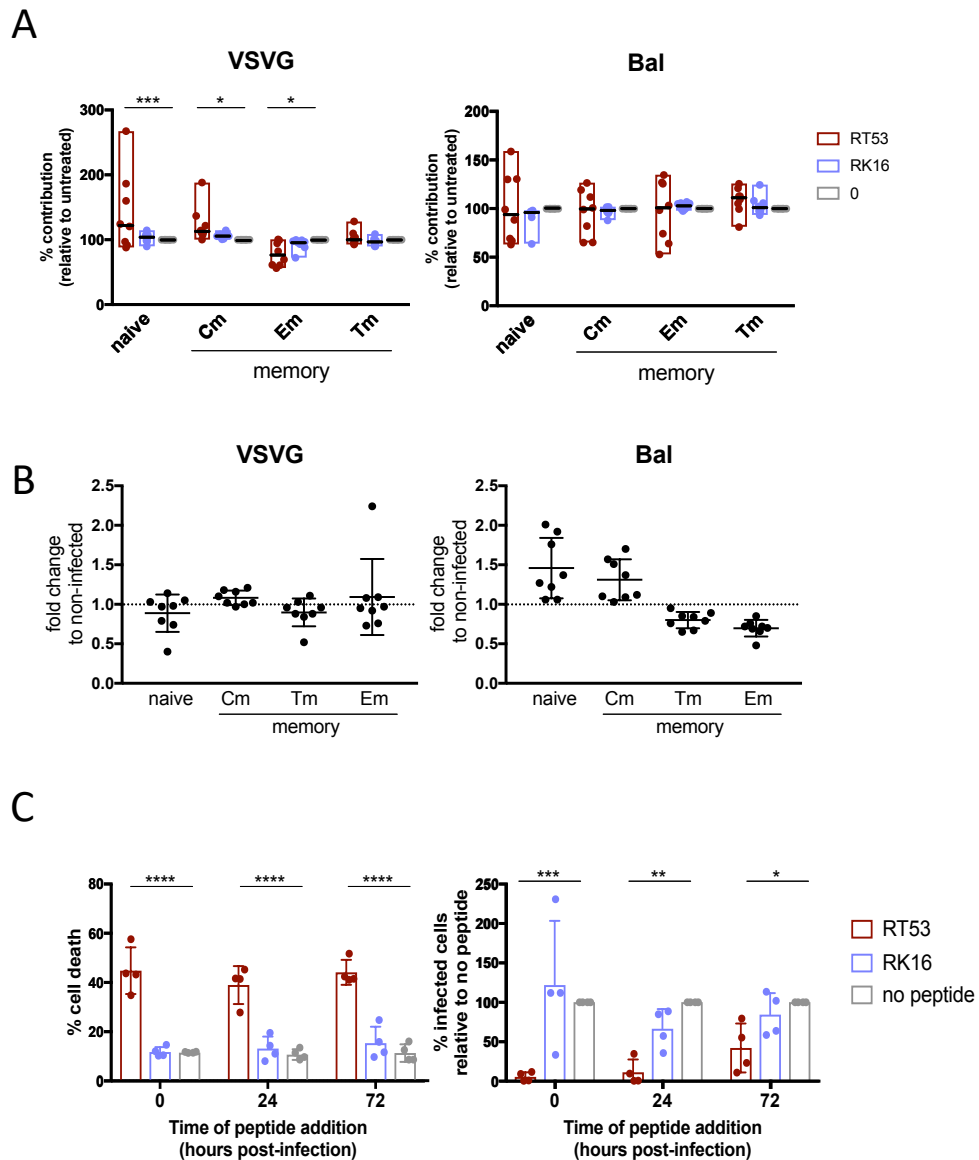
**CHAPTER 2:**  
**INVESTIGATION OF RT53 SPECIFICITY AND MECHANISM OF**  
**ACTION**

In Chapter 1 of this thesis manuscript, I described that AAC-11-derived peptides preferentially targeted HIV-1 susceptible CD4+ T cell population to cell death and left surviving cells resistant to productive infection. We observed that RT53, the most potent peptide in terms of cell death and anti-viral activity, displayed selectivity for highly differentiated, activated and metabolically active CD4+ T cell subsets. These characteristics have already been described as factors associated with permissibility of cells to HIV-1 infection. We also saw that RT53-mediated cell death was associated with caspase-2 activation. In this chapter, I attempt to further characterize RT53 cell type specificity and the mechanism of action.

### ***Extended insight into the enhanced sensitivity of wild type HIV-1 to the action of RT53***

We have shown that AAC-11 derived peptides induced cell death of CD4+ T cell subpopulations that are particularly susceptible to HIV-1 infection. However, infection with WT viruses was more sensitive to the action of the peptides than single cycle VSVG pseudotypes (Chapter 1, Fig 3). We have shown that treatment with RT53 depleted more differentiated cells while enriched the cultures in naïve CD4+ T cells (Chapter 1, Figure 5). We observed similar results when RT53 was added to cultures upon infection with VSVG-pseudotyped particles, showing that such infection did not alter the survival pattern of CD4+ T cell subsets. Indeed, infection with VSVG-pseudotyped particles did not alter the relative distribution of CD4+ T cells when compared to non-infected cultures (Fig 1A). In contrast, Bal infection, in the absence of peptide treatment, already resulted in the some depletion of Em and Tm subsets relative to non-infected control (Fig 1B), presumably due to HIV cytopathic effect. Moreover, we could not observe further change in the relative distribution of CD4+ T cell subsets upon treatment with RT53 in cells that had been exposed to Bal (Fig 1A). These results suggest that infection with wild-type HIV-1 may be modifying the pro-/anti-apoptotic intracellular balance of exposed cells. This is in agreement with previous observations that signaling through HIV-1 Env and Nef (absent in VSVG-pseudotyped HIV-1 particles) induces changes in key molecular regulators of apoptosis (Perfettini et al., 2004)(Perfettini et al., 2005)(Castedo et al., 2002)(Genini et al., 2000)(Cooper et al., 2013b)(Wolf et al., 2001b).

We next investigated cell death and HIV inhibitory capacity of RT53 when added at various time points post-infection with HIV-1 Bal (Fig 1C). RT53 potently blocked infection when added at 0, 24 or 72 hours post-infection. However while the amount of cell death induced by the peptide did not change (when compared to control cells at the same time points), RT53 was most effective in blocking Bal when added at the earlier time points. This indicated to us that RT53 1) may induce an extra block to infection with Bal in cells that survive treatment with the peptide and that this effect may be partially lost once the virus is integrated, and/or 2) Bal infection could be modifying infected cell survival to RT53 treatment, as hypothesized above, and this effect may wane overtime.



**Figure 1. Bal infection modifies cell survival to RT53 treatment.** **A.** Suboptimally activated CD4<sup>+</sup> T cells were infected with indicated HIV-1 viral strains and pulsed with 6  $\mu$ M RT53 or RK16. Cells were collected and phenotyped by flow cytometry on day 3 of infection. **B.** Relative contribution of CD4<sup>+</sup> T cell memory subsets to the total CD4<sup>+</sup> T cell pool on day 3 post-infection with indicated viral strains in the absence of peptide treatment. **C.** Activated CD4<sup>+</sup> T cells were infected with HIV-1 Bal and 6  $\mu$ M RT53 or RK16 were added at indicated hours post-infection. The amount of cell death and infection were quantified by flow cytometry on day 3 post-infection.

## ***Investigation of RT53 cell type specificity***

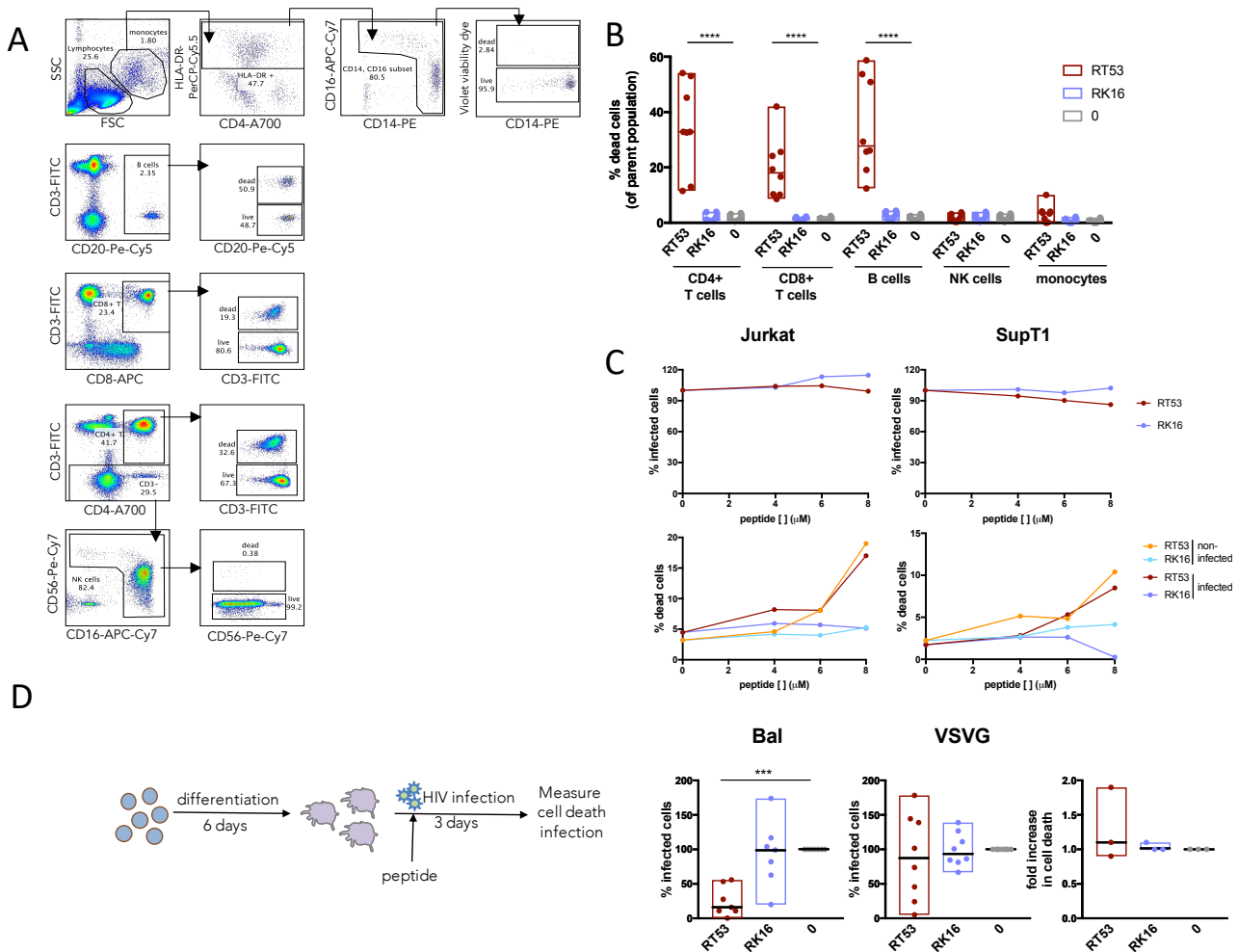
### ***RT53-induced mortality is not specific to CD4+ T cells***

Our previous results suggest that AAC-11 might be involved in the regulation of the CD4+ T cell response, and we hypothesize that the AAC-11 survival pathway may be also active in other blood cell fractions. We thus analyzed the effect of RT53-treatment on different subpopulations of peripheral blood mononuclear cells (PBMCs) *ex vivo*. We evaluated the levels of cell death in B cells, CD8+ and CD4+ T cells, NK cells and monocytes after 5 hour treatment with RT53 or RK16 (Fig 2A). RT53 caused mortality in B cells, CD4+ and CD8+ T cells but not in NK cells or monocytes (Fig 2B). Therefore, AAC-11 survival pathway targeted by RT53 may be important/specific for small lymphocyte development, maturation and survival but not for maintenance of other subsets.

Interestingly, RT53 treatment was not cytotoxic to CD4+ T cell lines Jurkat and SupT1 at 6 $\mu$ M peptide concentration, at significant cytotoxicity was observed in primary CD4+ T cells (Chapter 1, Fig S1), and only produced some cell death at 8 $\mu$ M. The impact on infection was, at best, very modest even at 8 $\mu$ M (Fig 2C). Thus, cancer cell lines are less sensitive to the action of RT53 than primary T cells, which is consistent with known overexpression of AAC-11 survival pathway in cancer cells. In addition the absence of significant effect of RT53 on infection levels in these cell lines is likely linked to the overall enhanced susceptibility of these cells to HIV-1 infection, perhaps related to their heightened metabolism.

The absence of dramatic cytotoxic effects of RT53 on monocytes made us wonder whether RT53 may cause mortality and inhibition of infection in macrophages, one of the major targets for HIV infection that could play an important role in HIV-1 persistence due to their longevity. To evaluate this, CD14+ monocytes were purified from human PBMCs and cultured for 6 days for differentiation into macrophages (Fig 2D). Cells were then infected with Bal or VSVG-pseudotyped single cycle HIV-1 and pulsed with 6  $\mu$ M RT53 or RK16. Infection and cell death were evaluated 3 days later. Similarly to the results observed in CD4+ T cells, RT53 resulted in

a drastic decrease in Bal infection of primary human macrophages but the effect on VSVG-pseudotyped viral particle infection was heterogeneous (Fig 2D). Interestingly, variable levels of cell death were observed. Therefore, RT53 is able to inhibit wild-type multi-cycle HIV-1 infection in primary macrophages. However, the sensitivity of monocytes and macrophages to RT53-induced cell death needs to be investigated in more detail.

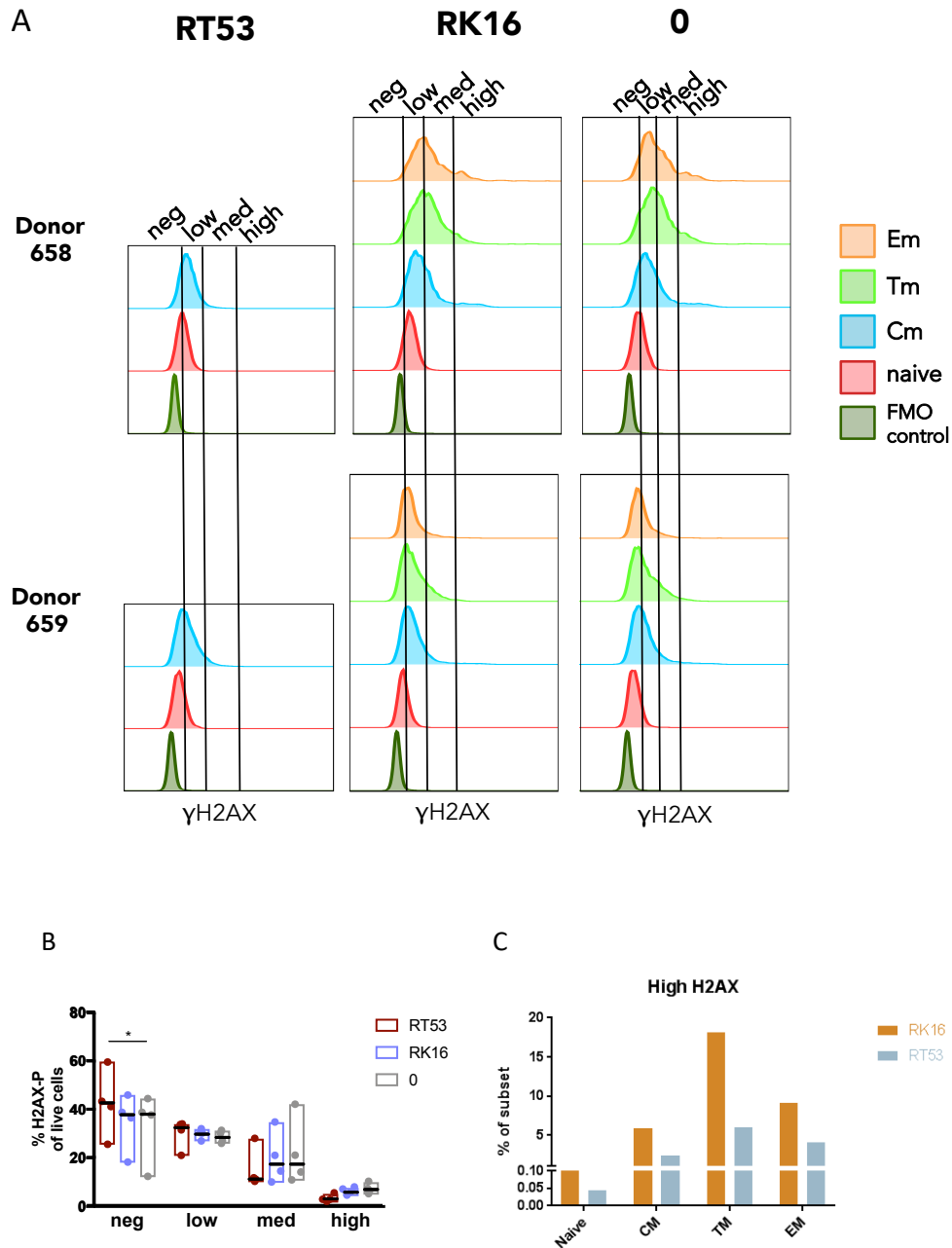


**Figure 2. RT53 selectively targets small lymphocytes to cell death and inhibits HIV-1 infection in macrophages.** **A.** Gating strategy for PBMC subsets. **B.** *Ex vivo* purified PBMCs were treated with 6  $\mu$ M RT53 or RK16 for 5 hours prior to flow cytometry staining **C.** Jurkat and SupT1 CD4+ T cell lines were infected with VSVG-pseudotyped HIV-1 particles and incubated with 6  $\mu$ M RT53 or RK16 for 3 days. % infected cells is normalized to infected cells not treated with peptides **D.** The effect of RT53 and RK16 on primary human macrophages infection and cell death. Min-max range and medians are indicated.



## ***DNA double strand breaks (DSB) as a marker of RT53-susceptible cell population***

Earlier in our experiments we observed a strong activation of caspase-2 in cells treated with RT53 (Chapter 1, Fig 4D). Caspase-2 activation is most known to trigger apoptosis in response to DNA damage (Miles et al., 2017). In this setting, *ataxia telangiectasia mutated* (ATM) is recruited to DSB, where it induces the formation of the PIDosome and activation of caspase-2 (Tinel and Tschopp, 2004). Upon activation, caspase-2 cleaves and activates various mediators of apoptosis. The presence of DSB in cells in non-cytotoxic conditions is linked to their cell cycle phase (ie DSB during DNA replication). Different CD4+ T cell subsets are known to exhibit different cycling states in the blood. Naïve cells are mostly non-cycling, followed by intermediate cycling in Cm and high cycling in Em cells (Thome et al., 2014). On the other hand, the presence of DSB in memory T cell subsets has also been linked to telomere shortening and predisposition to cell senescence in more differentiated memory subsets (Mitri et al., 2011). During HIV infection, DSBs are created during integration and are repaired by cellular DNA damage repair machinery. We, therefore, wondered if AAC-11 survival pathway might repress caspase-2 induced apoptosis in CD4+ T cell memory subsets with DSB acquired during DNA replication. We thus evaluated the proportion of cells with DSB in CD4+ T cells. We measured DSB by flow cytometric detection of H2AX histone phosphorylated at Ser139 (also known as  $\gamma$ H2AX), a common marker for DSB (Kuo and Yang, 2008). We reasoned that if RT53 triggers cell death mechanism in cells with DSB, we would see a selective depletion of a cell population expressing  $\gamma$ H2AX. Similarly to previous reports (Mitri et al., 2011), we observed higher expression of  $\gamma$ H2AX in Em and Tm cells followed by Cm and finally only a low level of  $\gamma$ H2AX in naïve cells (Fig 3A). We saw that CD4+ T cells with high expression of  $\gamma$ H2AX were preferentially depleted, whereas  $\gamma$ H2AX-low cells were enriched by RT53 treatment (Fig 3B) This correlated well with the preferential targeting of Em and Tm cells by RT53.  $\gamma$ H2AX<sup>high</sup> population was depleted in all subsets, even in naïve cells (Fig 3C). However, RT53 treatment also depleted Em and Tm cells with low levels of  $\gamma$ H2AX (Fig 3A). Thus, although cells with high levels of DSB appeared to be more sensitive to the action of RT53, DSB level was not an absolute marker for RT53 susceptibility.

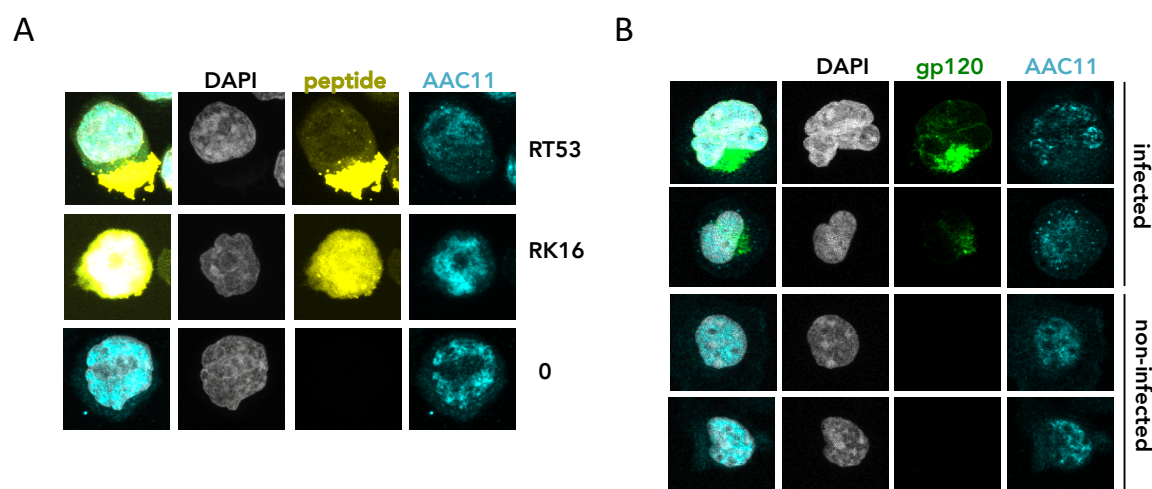


**Figure 3. RT53 preferentially targets cells with high proportion of DSB A.** The expression of  $\gamma$ H2AX across CD4+ T cell memory subsets upon treatment with RT53/RK16. Gating to identify positive population was performed using fluorescence minus one (FMO) control **B**. The amount of DSB in remaining total live cells after peptide treatment according to the levels of expression of  $\gamma$ H2AX **C**. Percent of  $\gamma$ H2AX<sup>high</sup> population among CD4+ T cell memory subsets after treatment with RT53 or RK16.

## ***Identification of cellular partners of RT53***

### ***RT53 partially localizes to the same cell compartment as AAC-11***

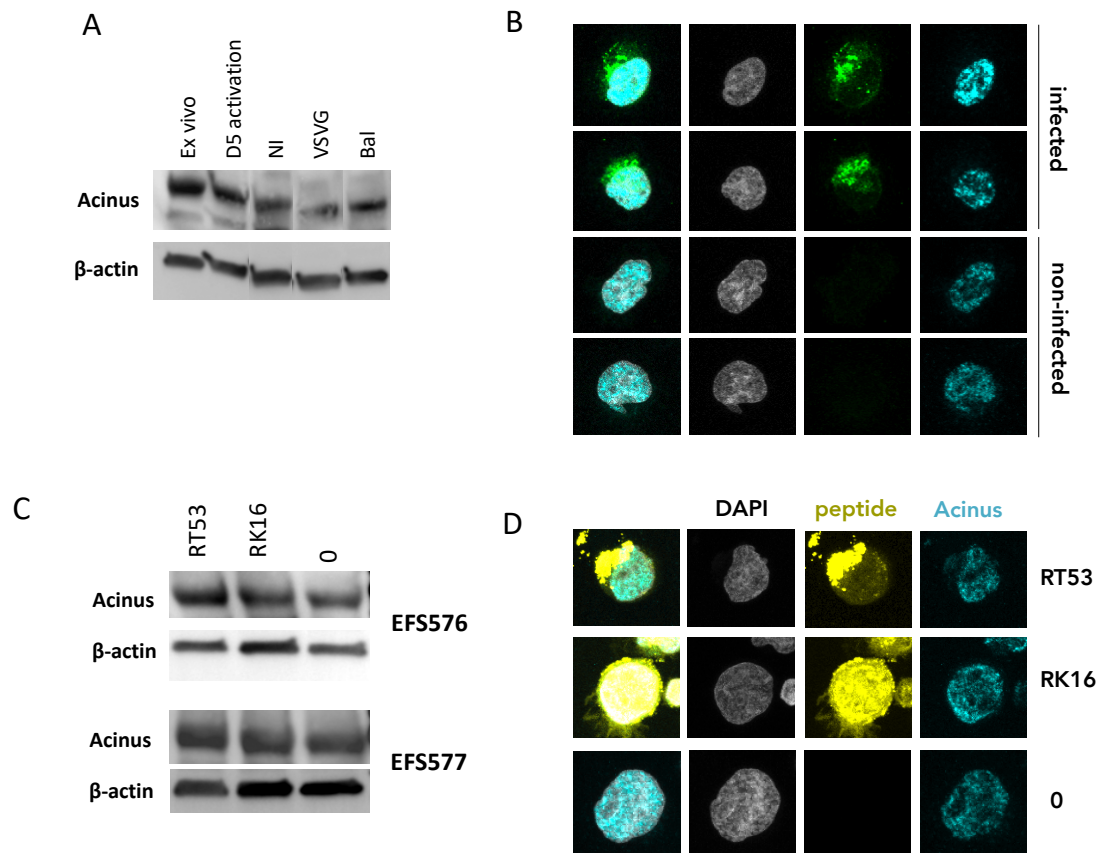
Although we have found that the transcript levels of AAC-11 in the different subpopulations of memory CD4+ T cells at the time of infection correlated with infection levels on day 3 post-infection in (Chapter 1, Fig 1), we did not observe any change in AAC-11 expression upon treatment with RT53, at least as measured by qPCR or western blot on bulk CD4+ T cells (Chapter 1, Fig S5). This is consistent with previously published observation that RT53's mechanism of action did not affect AAC-11 expression (Jagot-Lacoussiere et al., 2016) and it is probably related to interaction of RT53 with an AAC-11 partner that is expressed only in some of the cells upregulating AAC-11. In order to identify putative partners for RT53 we used fluorescently labelled peptides to determine their cellular localization. As expected RK16 penetratin was found mostly intracellularly (Fig 4A). In contrast, RT53 predominantly localized at the cell surface, clustered in what looked like a micro-domain or a membrane patch, probably retained there via interaction of AAC-11 LZ domain with a membrane partner. It is important to notice that RT53 was also found intracellularly, although at lower levels, where AAC-11 was localized itself. Infection or RT53 did not appear to change the cellular distribution of AAC-11, which remained mostly nuclear and some cytoplasmic (Fig 4A and B). These results suggest that one main RT53 interactor is located at the plasma membrane, although it is unclear whether the cytotoxic action of the peptides was related to the fraction retained at the membrane or the molecules that reached the intracellular compartments.



**Figure 4. Cellular distribution of RT53/RK16 and AAC-11 after peptide treatment and infection** A. Immunofluorescence of intracellular AAC-11 upon 2 hour treatment with 6  $\mu$ M RT53-Rd or RK16-Rd B. Immunofluorescence of intracellular AAC-11 in Bal infected (gp120 positive) and non-infected CD4+ T cells.

### ***Investigation of potential RT53 molecular partners***

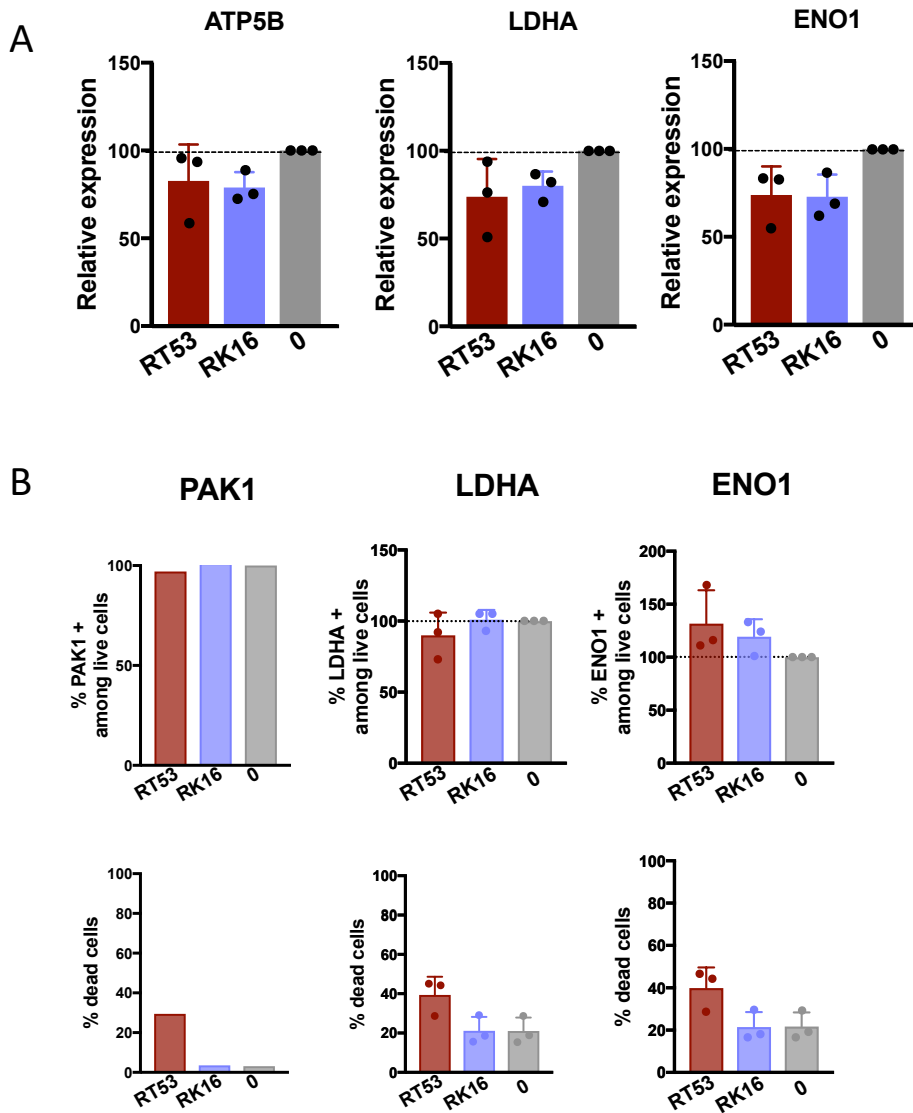
RT53 is expected to interact with an AAC-11 repressed partner thus unleashing a cell death cascade (Jagot-Lacoussiere et al., 2016). We, therefore, attempted to identify such a molecule. We first turned to Acinus, a known AAC-11 partner (Rigou et al., 2009), which mediates chromatin condensation (Sahara et al., 1999)(Hu et al., 2005) and nuclear DNA fragmentation (Joselin et al., 2006) during apoptosis. Acinus was also proposed to play a role in HIV-1 splicing (Rodor et al., 2016; Wojcechowskyj et al., 2013). We evaluated Acinus expression and localization upon infection and peptide treatment. We did not see any drastic changes in Acinus expression and localization upon cell activation or infection (Fig 5A, B). There were also no changes in Acinus expression or localization after cell treatment with RT53/RK16 (Fig 5C, D). These data suggest that RT53 does not act directly act through Acinus to induce cell death.



**Figure 5. Impact of infection and RT53/RK16 treatment on Acinus in CD4+ T cells** **A.** Expression of Acinus in *ex vivo* purified, activated (day 5), non-infected (NI) or infected (VSVG and Bal) (day 7 of infection) CD4+ T cells **B.** Acinus expression by immunofluorescence in infected (gp120 positive) and non-infected CD4+ T cells **C.** Expression of Acinus by Western blot upon 6  $\mu$ M RT53 or RK16 treatment in CD4+ T cells from two donors (EFS576 and EFS577) **D.** Expression of Acinus in RT53-Rd or RK16-Rd (6  $\mu$ M) treated CD4+ T cells.

Dr. Jean-Luc Poyet and his team used HuT78 T cell line to perform pull-down experiments followed by Mass Spectrometry to identify potential cellular partners of AAC-11-derived peptides. These experiments identified ATP synthase subunit  $\beta$  (ATP5B), L-lactate dehydrogenase  $\alpha$  chain (LDHA),  $\alpha$ -enolase (ENO1) and serine/threonine-protein kinase PAK1 (PAK1), among others. We thus set out to explore if any of these proteins could be the interacting partners of RT53 in our system. As before, we reasoned that if RT53 targets a cell

population expressing high levels of these proteins, this population would be preferentially depleted leaving surviving cells expressing lower levels of the protein of interest. We, therefore, evaluated the expression of these candidates first by PCR (Fig 6A) and then by flow cytometry (Fig 6B). We could not observe any consistent difference in the expression (at the gene or protein level) of these factors upon treatment with RT53 when compare to RK16 in experiments performed with cells from three donors. These results contrasted with the increased in RT53 induced cell mortality that was always observed in the same experiments. Like in the case of Acinus, our results, however, do not definitively exclude these candidates as potential interacting partners of RT53. It is possible that RT53 interacts with some or all of these molecules but only causes an effect in the downstream signaling pathway that is triggered only in a subset of cells. The cell surface and cytoplasm expression (and not total expression) of these proteins needs to be evaluated separately in the cell subsets that we know are more susceptible to the action of RT53.



**Figure 6. Changes in mRNA and protein expression of potential partners of RT53 peptide A.** mRNA expression of ATP5B, LDHA and ENO1 upon 5 hours treatment with 6  $\mu$ M RT53/RK16 **B.** Expression of indicated proteins evaluated flow cytometry upon 5 hours treatment with 6  $\mu$ M RT53/RK16.

# ***Role of ion homeostasis and cation channels in the action of RT53***

## ***Ca<sup>2+</sup> homeostasis plays a role in RT53 mechanism of action***

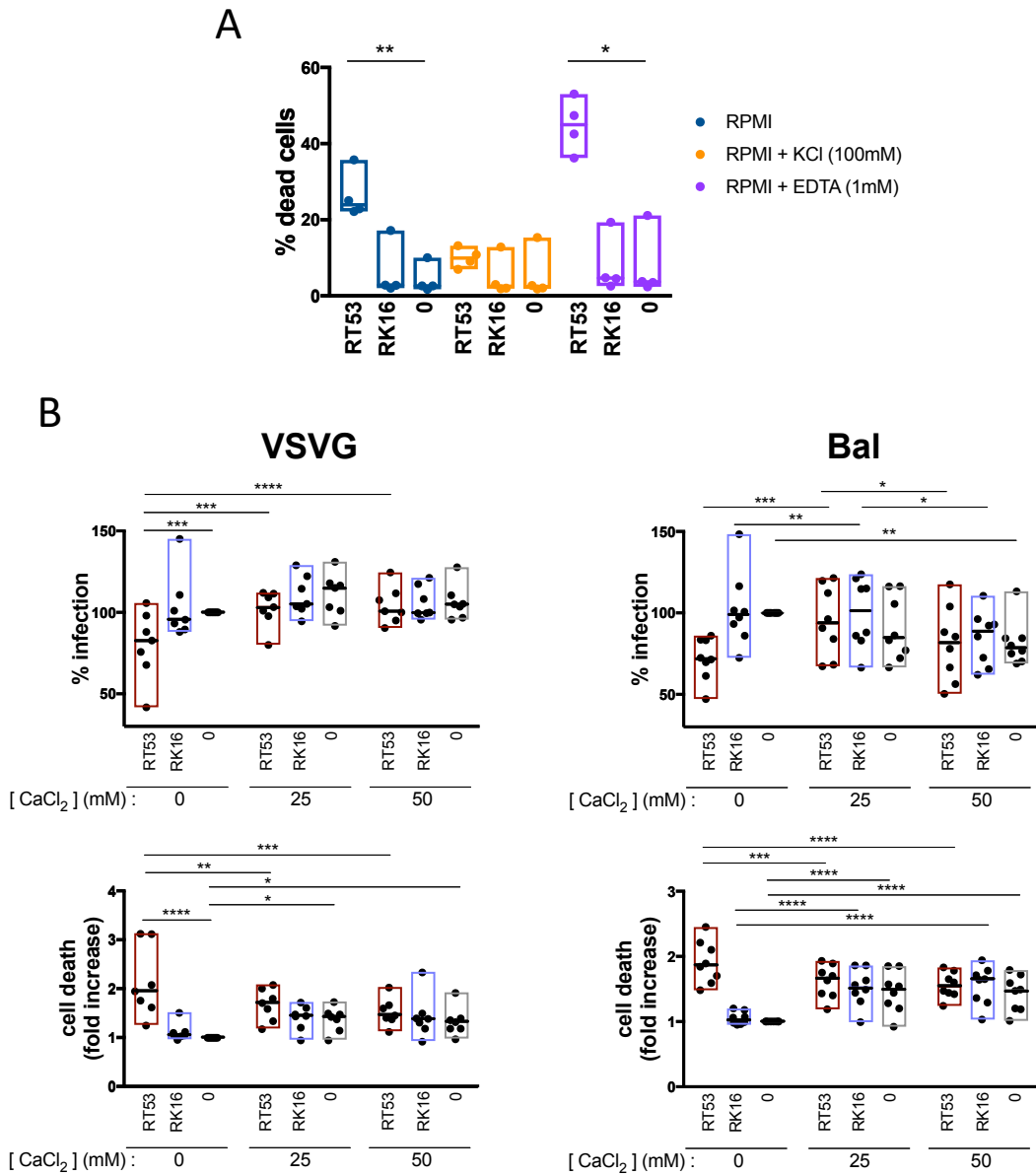
We had observed that K<sup>+</sup> efflux due to RT53 treatment in our cells and high concentrations of extracellular K<sup>+</sup> prevented RT53-induced cell death and preserved infectability of the cells (Chapter 1, Fig 4E). Several types of ion channels exist in T lymphocytes. Voltage-gated K<sup>+</sup> channels (K<sub>v1.3</sub>) are activated in response to PM depolarization (inside of the cell becoming more positive) due to Ca<sup>2+</sup> influx in response to TCR stimulation, which leads to K<sup>+</sup> efflux from the cell to restore resting membrane potential. K<sub>Ca3.1</sub> channels also mediate K<sup>+</sup> efflux, but are activated by the rise in cytoplasmic Ca<sup>2+</sup>. Ca<sup>2+</sup> release-activated Ca<sup>2+</sup> (CRAC) channels are activated in response to depletion of ER Ca<sup>2+</sup> stores and lead to Ca<sup>2+</sup> influx (Cahalan and Chandy, 2009). Transient receptor potential cation channel subfamily M member 4 (TPRM4) is a cation channel that is more permeable to Na<sup>+</sup> than Ca<sup>2+</sup>. It is activated by Ca<sup>2+</sup> influx following TCR stimulation and results in Na<sup>+</sup> influx thus depolarizing the membrane and limiting Ca<sup>2+</sup> influx (Feske et al., 2015b).

We previously demonstrated that RT53 causes a decrease in intracellular K<sup>+</sup> concentration (Chapter 1, Fig 4A), which we linked to RT53-mediated cell death. Ca<sup>2+</sup> ions were also reported to activate molecular pathways of apoptosis. For example, cytoplasmic Ca<sup>2+</sup> overload was reported to stimulate apoptosis via opening of MPTP (Kondratskyi et al., 2015). To determine if Ca<sup>2+</sup> could also be linked to RT53-activated cell death pathway, we incubated cells for 5 hours in the presence of 6μM RT53 or RK16 ± 1mM ethylenediaminetetraacetic acid (EDTA) to chelate Ca<sup>2+</sup> ions from the extracellular medium. The presence of EDTA in the extracellular medium further increased susceptibility of cells to RT53-mediated cell death (Fig 7A). We next tested if elevated extracellular Ca<sup>2+</sup> could protect cells from RT53-triggered cell death. Indeed, similarly to what we observed with KCl, the addition of exogenous CaCl<sub>2</sub> decreased cell death and preserved susceptibility of CD4<sup>+</sup> T cells to HIV-1 BaL and VSVG-pseudotyped HIV-1 infected particles (Fig 7B). The effect of CaCl<sub>2</sub> was dose dependent and cell death reached baseline at 50mM CaCl<sub>2</sub> (half of the molar ratio that was needed to suppress cell death with



KCl). Interestingly, we observed that cell death directly induced by infection with HIV-1 Bal, but not with VSVG-pseudotyped particles, increased with CaCl<sub>2</sub> concentrations (Fig 7B). This was accompanied by a decrease in infection with HIV-1 Bal at higher (50mM) CaCl<sub>2</sub> concentration in the absence of peptides. These results may reflect previously described changes in ion homeostasis induced by HIV Env and Nef proteins to enhance HIV replication (Séror et al., 2011)(Voss et al., 1996).

An inter-connected network of ion channels in the cell's membranes tightly regulates signaling and membrane potential (Bose et al., 2015). Cell hyperpolarization (the inside of the cell becoming more negative) by K<sup>+</sup> efflux is known to trigger influx of Ca<sup>2+</sup> or Na<sup>+</sup> through TPRM channel to restore resting membrane potential of around -70mV (Bose et al., 2015). It is thus possible that activation of inward rectifying cation current of Ca<sup>2+</sup> or Na<sup>+</sup> (not tested here) could act as a mechanism to protect the cell against hyperpolarization-driven cell death. RT53 could thus be interacting with K<sup>+</sup> ion channels to drive them into open conformation to induce K<sup>+</sup> efflux or K<sup>+</sup> efflux may be the result of RT53 directly inducing membrane pores upon interaction with another membrane partner (as already suggested in (Jagot-Lacoussiere et al., 2016)).



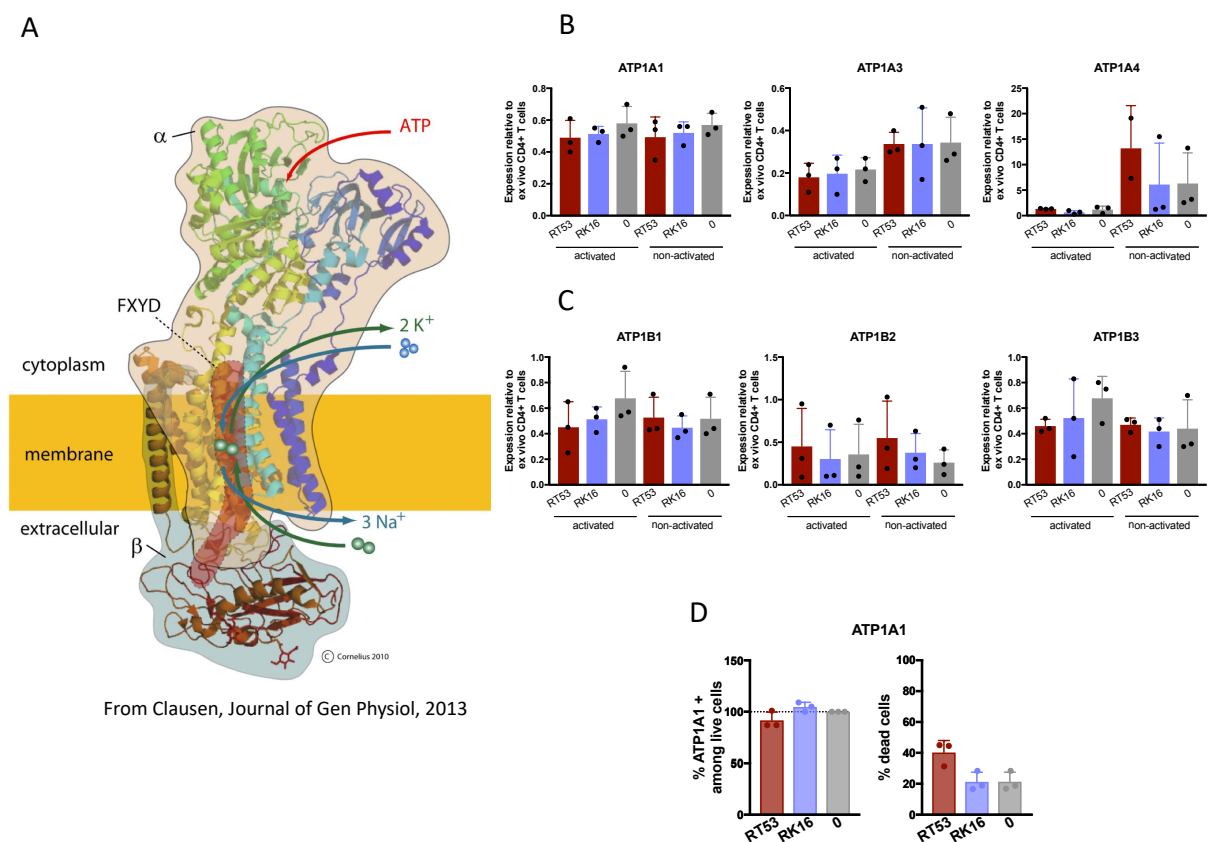
**Figure 7.  $\text{Ca}^{2+}$  homeostasis plays a role in cell susceptibility to RT53-mediated cell death and infection** **A.** CD4<sup>+</sup> T cells were incubated with 6  $\mu\text{M}$  RT53 or RK16 for 5 hours in the presence of 100mM KCl or 1mM EDTA. Cell death was evaluated by flow cytometry **B.** Elevated extracellular  $\text{Ca}^{2+}$  rescues cells from RT53-mediated cell death and inhibition of infection. Activated CD4<sup>+</sup> T cells were infected with VSVG-HIV-1 or Bal for 3 days and then incubated with 6  $\mu\text{M}$  RT53 or RK16 for 5 hours in the presence of the cell medium containing various concentrations of  $\text{CaCl}_2$ . Cell death and infection were quantified by flow cytometry.

## ***Investigation of Na<sup>+</sup>/K<sup>+</sup> ATPase (NKA) subunit expression upon peptide treatment***

NKA ensures high intracellular K<sup>+</sup> and low intracellular Na<sup>+</sup> concentrations by pumping 2 K<sup>+</sup> ions inside and 3 Na<sup>+</sup> ions outside the cell. This is accomplished via energy derived from the hydrolysis of an ATP molecule (Banerjee and Xie, 2016) (Fig 8A). Thus, alternatively to RT53-mediated opening of K<sup>+</sup> channels, AAC-11-derived peptides could inhibit NKA function ultimately reducing intracellular K<sup>+</sup> concentration. NKA is a transmembrane heterodimer composed of 1  $\alpha$  and 1  $\beta$  subunit. All subunits have transmembrane topology. The  $\alpha$  subunit has a large cytoplasmic domain, which contains an ATP binding pocket. The  $\beta$  subunit, on the other hand, contains a large extracellular domain. A small transmembrane protein called the  $\gamma$  subunit is also found associated with NKA but will not be considered here because most of it is embedded in the membrane (Fig 8A). There are 4 known isoforms of the  $\alpha$  subunit (ATP1A1-4) and 3 of the  $\beta$  subunit (ATP1B1-3). NKA inhibition by cardiac glycosides such as digitoxin, digoxin or ouabain was already implicated in the inhibition of several alpha viruses including Chikungunya, Ross River virus and Sindbis virus at post-entry level (Ashbrook et al., 2016). Recently, NKA also became implicated in HIV-1 infection. Expression of CD298, isoform 3 of NKA  $\beta$  subunit was strongly correlated with permissibility of primary CD4<sup>+</sup> T cells to infection (Rato et al., 2017), although if CD298 expression in HIV-1 susceptibility is related to its function as a subunit of NKA is unclear.

Interestingly preliminary results by JL Poyet lab point to ATP1A4 as one possible interactor with AAC-11-derived peptides. We thus analyzed the expression of various isoforms of NKA  $\alpha$  and  $\beta$  subunits in primary CD4<sup>+</sup> T cells (Fig 8B). As before, we reasoned that if RT53 is targeting one of the NKA subunits, we would see a decrease in its expression due to selective death of cells expressing high levels of this subunit. The mRNA expression of isoforms 1, 3 and 4 (ATP1A1, ATP1A3 and ATP1A4 respectively) but not 2 (ATP1A2) of the  $\alpha$  subunit was detected in CD4<sup>+</sup> T cells. The mRNA expression of all three isoforms of the  $\beta$  subunit was detected (Fig 8C). The expression of ATP1A3 and ATP1A4 decreased with CD4<sup>+</sup> T cell activation while the expression of ATP1B3 increased (Fig 8B and C). However, none of the subunits mRNA expression decreased consistently across donors upon RT53 treatment. This

provided us with limited information on the regulation of NKA upon RT53 treatment, however, as NKA is known to have a complex post-translational regulation. Therefore, we next proceeded to evaluate the expression levels of ATP1A1 on a protein level by flow cytometry. We saw some decrease in the expression of ATP1A1 protein (Fig 8D) although as before more thorough analyses of the expression of ATP1A1 at the CD4+ T cell subset level will be necessary to understand the implication of this protein in the action of RT53. The expression of the rest of the subunits is ongoing. Additionally, to identify if RT53 interacts with any of the NKA subunits on the cell surface, subunit-specific immunofluorescence experiments to see potential RT53 and NKA subunit co-localization need to be performed.



**Figure 8. Expression of  $Na^+/K^+$  ATPase subunits with CD4+ T cell activation and treatment with RT53 or RK16** **A.** Structure of NKA subunits and their associated function **B.** Cells were subjected to 6  $\mu$ M RT53/RK16 for 5 hours and mRNA/protein levels were then evaluated by PCR or flow cytometry respectively. Changes in NKA  $\alpha$  subunit and **C.** in NKA  $\beta$  subunit mRNA expression upon CD4+ T cell activation and peptide treatment **D.** Expression  $\alpha$  subunit isoform 1 upon peptide treatment evaluated by flow cytometry.

## Materials and Methods

### Peptides, antibodies and probes

Peptides (Proteogenix) were received as dry powder and reconstituted with water for use.

Peptide sequences are as follows.

RT53: RQIKIWFQNRRMKWKKAKLNAEKLDKFKIRLQYFARGLQVYIRQLRLALQGKT,

RT39: RQIKIWFQNRRMKWKKLQYFARGLQVYIRQLRLALQGKT,

RL29: RQIKIWFQNRRMKWKKYFARGLQVYIRQL,

RQ26: RQIKIWFQNRRMKWKKLQYFARGLLQ,

RK16: RQIKIWFQNRRMKWKK.

Antibodies and dyes used for flow cytometry and FACS sorting are as follows: LIVE/DEAD™ Violet Viability dye (ThermoFisher), CD3-PE (clone SK7, Biolegend), CD4-A700 (clone OKT4, eBioscience), CD45RA-APC-Cy7 (clone HI100, Biolegend), CCR7-Pe-Cy7 (clone GO43H7, Biolegend), CD27-APC (clone M-T271, Mylteniy), CD25-PE-Dazzle594 (clone M-A251, Biolegend), HLA-DR-PerCP-Cy5.5 (clone G46-6, Biolegend), CCR5-PE (clone 3A9, BD), p24-FITC (clone KC57, Coulter), CD14-PE (clone TUK4, Mylteniy), CD3-FITC (clone SK7, Biolegend), CD56-PE-Cy7 (clone N901, Beckman Coulter), HLA-DR-PerCP-Cy5.5 (clone G46-6, Biolegend), CD25-PC5 (clone B9E9, Beckman Coulter), CD16-APC-H7 (clone 3G8, BD), CD4-A700 (clone OKT4, eBioscience), CD8-V500 (clone RPA-T8, BD),  $\gamma$ H2AX-A647 (clone 20E3, Cell Signaling Technology®), PAK1-A488 (clone EP795Y, abcam). The following primary and fluorochrome conjugated secondary antibody pairs were used for flow cytometry: rabbit anti-ATP1A1 IgG (Genscript, Cat A01483), rabbit anti-ENO1 (abcam, Cat ab52488) and rabbit anti-LDHA (abcam, Cat ab52488) all followed by anti-rabbit IgG-A647 (LifeTechnologies, Cat A31573). Flow cytometry acquisition was performed on BD LSRII.

### Cell lines

Jurkat and SupT1 cell lines were cultured in RPMI-1640 containing Glutamax (ThermoFisher), 10% fetal bovine serum (FBS) and penicillin-streptomycin (ThermoFisher) (100 U/ml). Cells were split twice a week and used at a concentration of  $10^6$  cells/ml for peptide assays.

### **Isolation and culture of primary human CD4+ 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). Blood was overlaid on Ficol (EuroBio) at a ratio of 2:1 v/v blood to Ficol and centrifuged at 1,800 rpm for 30 minutes at a minimum acceleration/deceleration to obtain peripheral blood mononuclear cells (PBMCs). CD4+ T cells were then purified from PBMCs by negative selection using StemCell EasySep™ Human CD4+ 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 (Myltenyi) (100U/ml) (thereafter referred to as culture medium) at  $10^6$  cells/ml in 37° degree, 5% CO<sub>2</sub> humidified incubator. Cells were suboptimally activated with soluble anti-CD3 (clone UCHT-1) (Biolegend) for 5 days prior to infection or analysis as previously described (Sáez-Ciri3n et al., 2011).

### **Generation of human monocyte-derived macrophages**

CD14+ monocytes were purified from human PBMCs by EasySep™ Human CD14 Positive Selection Kit (StemCell) and differentiated as previously described (David et al., 2006). Briefly, CD14+ monocytes were placed on Lumox Teflon bottom plates (Starstedt, Cat 94.6077.305) at  $10^6$  cells/ml in RPMI-1640 medium containing 2 mM L-glutamine (LifeTechnologies), 100U/ml Penicillin, 100 µg/ml Streptomycin (LifeTechnologies), 10mM sodium pyruvate (LifeTechnologies), 10 mM HEPES (LifeTechnologies), 1% MEM vitamins (LifeTechnologies), 1% non-essential amino acids (LifeTechnologies), 50 µM β-mercaptoethanol (LifeTechnologies) (MDM medium) and 15% human AB serum and cultured for 6 days. Differentiated macrophages were harvested by gentle scraping, counted and resuspended at  $10^6$  cells/ml in MDM medium supplemented with 10% FBS for further experiments.

### **Western Blot**

$10^6$  cells was pelleted and stored as dry pellet in -80°C until use. The cell pellet was lysed in a lysis buffer (150mM NaCl, 50mM pH 7.4 Tris, 1mM pH 8 EDTA, 1% Triton X-100, containing 1

tablet of complete EDTA-free protease inhibitor cocktail (Sigma) per 10ml in water) for 20min on ice with occasional vortexing. 30 µg total protein was loaded on a 4-15% gradient SDS-PAGE gel (Mini Protean TGX Stain-Free gel, Biorad) previously denatured in the presence of 20% v/v loading buffer (LifeTechnologies) and 1% v/v β-mercaptoethanol for 10min at 95°C, and run at 120V for 1 hour. Samples were then transferred on PVDF membrane (Dutscher Dominique) previously activated in 100% ethanol for 1 minute in a transfer buffer (25 mM Tris, 192 mM glycine, 20% ethanol, pH 8.3) at 95V (300mA) for 40 min. The membrane was blocked in 5% BSA in Tris-Buffered Saline 0.1% tween-20 (TBST) over night at 4°C. The membrane was blotted with anti-Acinus (Cell Signaling, 4934) at 1/2,500 dilution overnight at 4°C in TBST, washed 3 times with TBST and incubated with anti-rabbit HRP (Sigma, A0545) at 1/1,000 dilution in TBST for 45 minutes at RT. Membrane was developed with ECL reagent (Thermo Scientific).

### **Immunofluorescence microscopy**

Activated CD4<sup>+</sup> T cells were incubated with 6µM RT53-Rhodamine [Rhodamine-RQIKIWFQNRRMKWKKAKLNAEKLKDFKIRLQYFARGLQVYIRQLRLALQGKT] or RK16-Rhodamine [Rhodamine-RQIKIWFQNRRMKWKK] (Proteogenix) for 2 hours. Cells were then washed twice with PBS, immobilized on poly-lysine coated coverslips, fixed with 4% paraformaldehyde for 10 minutes at room temperature (RT), neutralized with 50mM NH<sub>4</sub>Cl for 10min at room temperature and washed twice with PBS. Cells were then permeabilized with 0.1% Triton-X 100 in PBS for 5 min at RT. 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) 1/200 dilution for 1hr at RT followed by secondary Ab (Invitrogen, A31573) 1/400 dilution for 45min at RT. Intracellular staining for gp120 was performed with anti-gp120 (clone 2G12, NIH AIDS Reagent Program, Cat 1476) 1/100 dilution for 1hr at RT followed by anti-human IgG-A488 (Jackson, Cat 709-545-149) 1/400 for 45min at RT. Intracellular staining for Acinus was performed using anti-Acinus Ab (Cell Signaling, Cat 4934) 1/100 dilution for 1hr at RT followed by anti-rabbit IgG-A647 (LifeTechnologies, A31573) 1/400 dilution for 45 min at RT. Coverslips were then washed, stained with DAPI for 15min at RT and mounted using Fluoromont G (ThermoFisher) mounting medium.

## RT-PCR

Cells were collected by centrifugation, lysed in RA1 buffer (Macherey-Nagel) and stored at -80°C until RNA extraction. RNA was purified using RNA isolation kit (Macherey-Nagel) as per manufacturer's instructions. RNA was then converted to cDNA using SuperScript II (Thermo Fisher) as per manufacturer's instructions. RT-PCR was performed on 7500 Real Time PCR System (Applied Biosystems) using TaqMan Universal Master Mix II, with UNG (Thermo Fisher) with the following probe (FAM-MGB)-primer pairs: ATP1A1 (Hs00167556\_m1), ATP1A2 (Hs00265131\_m1), ATP1A3 (Hs00958036\_m1), ATP1A4 (Hs00380134\_m1), ATP1B1 (Hs00426868\_g1), ATP1B2 (Hs01020302\_g1), ATP1B3 (Hs00740857\_mH), ENO1 (Hs00361415\_m1), LDHA (Hs01378790\_g1), ATP5B (Hs00969569\_m1), AAC-11 (Hs00362482\_g1).  $\Delta C_t$  was calculated using BECN1 (Hs01007018\_m1) and  $\Delta\Delta C_t$  was calculated using reference *ex vivo* purified CD4+ T cell sample.



## DISCUSSION

The search for intracellular factors that allow the establishment of efficient HIV viral infection is an active area of investigation today. Molecular pathways responsible for cell activation, cell cycle progression and metabolism are now clearly shown to be used by HIV for its replication. These pathways provide favorable intracellular environment for all stages of HIV infection with particular impact on RT, integration and transcription. Cell activation is accompanied by increase in mitochondrial respiration and glycolysis, which together provide biosynthetic substrates, such as nucleotides, amino acids and carbohydrates for most stages of viral replication (RT, transcription and translation, protein glycosylation, etc). Open chromatin conformation and active gene transcription associated with activated and cycling cells allow efficient viral integration and transcription.

Although poorly understood, cell survival pathways are equally important for HIV propagation and persistence. Despite viral cytotoxicity seen *in vitro*, productively infected cells persist in culture due to mostly uncharacterized survival mechanisms. Additionally, infected cells with long half-life (particularly Tscm, Cm CD4+ T cells and macrophages) survive *in vivo*, despite massive cell death observed in primary infection, and contribute to the HIV reservoir. Today, several key questions remain unresolved: how do HIV infected cells survive? Does the virus induce survival pathways and if so which ones? Which pre-existing cellular survival mechanisms are used by the virus? And how the survival pathways used by HIV interact with other known intracellular HIV dependency factors?

In this work, we identified AAC-11-dependent anti-apoptotic pathway to be targeted by HIV to establish productive infection. Disruption of this pathway via the competitive antagonism of AAC-11 with the peptides derived from its  $\alpha$ 18 helix containing the LZ domain caused selective death of cells that are preferentially targeted by HIV-1 for productive infection. These results provide, therefore, a proof of concept that selective targeting of intracellular survival factors can lead to elimination of HIV target cells. This work also revealed the interaction of AAC-11 survival pathway with cellular differentiation, activation and metabolism, a combination of which allowed for efficient viral replication, and elimination of which blocked productive infection.

In this work, we observed the infectability of CD4+ T cell memory subsets at various differentiation stages (naïve<Cm<Tm<Em) to correlate with the proportion of cell death seen in each subset due to the AAC-11-derived peptide treatment. The susceptibility of each subset to cell death could be explained by intrinsic balance of intracellular pro- and anti-apoptotic factors characteristic of this subset. Indeed, memory T cells are thought to differ in their regenerative potential and longevity. Naïve T cells are known to be longer-lived than their memory counterparts (Macallan et al., 2017). Tscm and Cm cells are thought to be superior to other memory subsets in their ability to self-renew and sustain long-term survival (Gattinoni et al., 2011)(Lugli et al., 2013) (Mahnke et al., 2013). Em and Tm cells, on the other hand, are programmed for efficient effector responses and are relatively primed for apoptosis (Macallan et al., 2017). Therefore, the precarious viability of Em and Tm cells could depend on several anti-apoptotic factors such as AAC-11 among the overall pro-apoptotic intracellular milieu. Hence, the inhibition of these factors by treatments such as RT53 could easily launch cell death program in these subsets.

In this and our previous work (Valle-Casuso, submitted, See Annex 2) we observed that Em and Tm subsets were preferentially infected with both VSVG-pseudotyped HIV-1 particles and wild-type Bal HIV-1 (Chapter 1, Fig 5B). We previously showed (Valle Casuso, submitted, Annex 2) that this preference is at least in part related to the enhanced metabolic activity of this subset relative to other CD4+ T cell memory cells. In the current work, we identify that a particular combination of anti-apoptotic pathways (we identified one node of this network, AAC-11, but other components remain to be explored) in some cells of this subpopulation could also be contributing to the favourable intracellular milieu for HIV. On the other hand, Em and Tm cells are also most prone to HIV-1 induced cytotoxicity (Chapter 2, Figure 1B). Therefore, how some Em and Tm cells carrying the virus succeed to survive is an interesting and important question. *In vivo*, however, the relative contribution of Cm cells to the HIV reservoir is currently thought to be dominant over other CD4+ T cell subsets (Chomont et al., 2009b). Indeed, Cm subset is known to have higher survival and self-renewal capacity over other memory subsets. Therefore, while Em and Tm could be preferentially infected, long

term survival of Cm cells could contribute to its higher contribution to the HIV reservoir *in vivo*. Interestingly, it has been recently suggested that Em differentiate into Cm cells (Youngblood et al., 2017). Thus, AAC-11 dependent anti-apoptotic program of Em cells could give rise to long lived Cm cells harboring HIV-1 reservoir *in vivo*.

Our work further underlines the parallelism between cells susceptible to HIV infection and cancer cells, as well as demonstrates the potential usefulness of anti-cancer approaches as an anti-HIV strategy. The major hallmarks of cancer closely reflect well-established intracellular requirements for efficient HIV infection. These hallmarks include chronic proliferation (ie constant progression through the cell cycle and cell growth) accompanied by increasing metabolic demands, evasion of apoptosis and genomic instability (Hanahan and Weinberg, 2011). AAC-11 is known to be important for cancer cell survival and was already used as a proof of concept treatment for the elimination of malignant cells *in vitro* and *in vivo* (Jagot-Lacoussiere et al., 2016). In our study, we observed that counteracting AAC-11 survival pathway lead to the elimination of cells with characteristics most closely resembling those of cancer hallmarks (highly activated, metabolic cells with high degree of DSB), despite being non-malignant.

Cancer cells are known to upregulate Glut-1 transporter and aerobic glycolysis known as the Warburg effect (Hanahan and Weinberg, 2011). Indeed, high levels of radiolabelled glucose utilization is now used to non-invasively visualize tumors *in vivo* using positron emission tomography. HIV-1 also preferentially infects CD4+ T cells with high level of glycolysis and the block in infection is observed with inhibition of glucose utilization (Valle Casuso, 2018). In our experiments, we observed that RT53 preferentially targeted highly metabolically active cells. This indicates that AAC-11 survival pathway may be most active in cells with most active metabolism. Indeed, the link between metabolic and cell survival processes is well known (Green et al., 2014). Metabolic checkpoints are crucial in cell fate decisions and can tip the balance towards life or death during noxious stimuli such as RT53 by activation/suppression of cell death or survival effectors. Nutrient availability is sensed by central regulator of metabolism and cell survival, mTOR complex. During starvation, inhibition of mTORC1

promotes apoptosis (Green et al., 2014). On the other hand, active mTORC1 drives metabolic processes and promotes cell survival (Galluzzi et al., 2013). Interestingly, it has been reported that cells undergoing enhanced rates of glycolysis are able to survive intrinsic apoptosis due to the release of the oxidized form of cytochrome c from the mitochondria, which is unable to activate caspases (Vaughn and Deshmukh, 2008) (Green et al., 2014). Could highly glycolytic cells targeted by HIV-1 use survival pathways induced in such cells, and if so, which are these pathways? Intriguingly, activation of caspase 2 has already been shown to be linked to cellular metabolism. Suppression of caspase 2 activity was observed by the intermediates of pentose phosphate pathway (PPP) in *Xenopus laevis* oocyte through its inhibitory phosphorylation (McCoy et al., 2013) (Nutt et al., 2005). Therefore, AAC-11 pathway could perhaps suppress apoptosis in highly glycolytic cells by repressing caspase 2 and other apoptotic effectors and this repression could be lifted upon RT53 treatment.

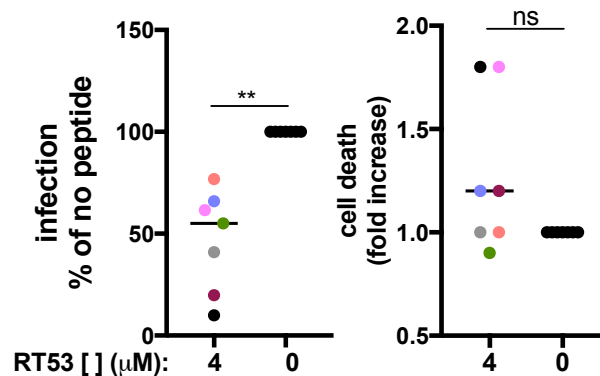
Chronic replication and avoidance of cell death are other hallmarks of cancer, which closely parallel HIV intracellular requirements. Many tumor suppressor molecules implicated in cell cycle and death decisions are now also identified as HIV-1 restriction factors. For example, p53 is a major tumor suppressor, which is stabilized by the action of caspase 2 in response to compromised genomic integrity (Oliver et al., 2011). It acts as a central decision point towards cell cycle arrest or apoptosis during genomic or metabolic insults (Kruiswijk et al., 2015)(Simabuco et al., 2018) and is inhibited in many cancers (Kruiswijk et al., 2015). Similarly to its role in suppressing cancer, p53 was reported to be an HIV-1 restriction factor through inhibition of its transcription (Mukerjee et al., 2010). Additionally, p53 downstream target p21, also strongly implicated in cancer through its regulation of cell cycle checkpoints (Georgakilas et al., 2017), has been characterized by us and others to be an HIV-1 restriction factor (Allouch et al., 2014; Pauls et al., 2014) (Badia et al., 2016)(Valle-Casuso et al., 2017).

P53 and its downstream targets act as genome guardians (Georgakilas et al., 2017), which, as mentioned above, are often disabled in cancer cells allowing cell survival and proliferation despite genomic instability, another key hallmark of cancer. Cancer provoking mutations often stem from the telomere shortening and associated DNA damage and chromosome

rearrangement events (Hanahan and Weinberg, 2011). Interestingly, subsets of memory T cells follow a gradient of telomere shortening and senescence-related proliferative defects that reflects their differentiation status (Mitri et al., 2011). Em and Tm cells are most primed for senescence and apoptosis due to their high expression of DSB related to telomere shortening, to which DNA repair machinery is rapidly recruited (Mitri et al., 2011). In our work, we observed that Em and Tm subsets that displayed the highest proportion of DSB were preferentially depleted by RT53 (Chapter 2, Fig 3). However, we cannot distinguish if the disappearance of cells enriched in DSB upon RT53 treatment is a cause or only a consequence of cell death in our experiments. Similarly to cancer, DSB are important for HIV life cycle. Viral integration critically relies on DNA repair machinery that is recruited to DSBs created by viral integrase (Daniel, 2006) (Daniel et al., 2004). Despite this, however, a subset of cells was reported to undergo apoptosis in response to HIV-1 integration (Cooper et al., 2013b). Therefore, successful viral integration and persistence critically rely not only on cellular DNA repair machinery but also on anti-apoptotic survival pathways in cells that are preferentially targeted by the virus.

We also saw that RT53 inhibited VSVG-pseudotyped HIV-1 with less potency than wild-type Bal (Chapter 1, Fig 3). This could be due to entry of these two viral strains to different subsets of CD4+ T cells. VSVG-pseudotyped HIV-1 particles are thought to enter equally into different T cell subsets (Dai et al., 2009). Wild-type HIV-1, on the other hand, enters preferentially into memory than naïve cells (Dai et al., 2009) due to their higher expression of HIV-1 co-receptor. Indeed, we did observe a decrease in CCR5<sup>high</sup> cells in our cultures upon peptide treatment, but this did not fully account for the observed effect. Multiplicity of viral cycles of wild-type Bal HIV-1 could also be responsible for stronger RT53-mediated inhibition. In this case, inhibition of Bal at early stages of replication in RT53 treated cells would produce higher differences with RK16 treated and untreated cells, which accumulated many viral re-infection cycles. VSVG-pseudotyped virus, on the other hand, would only be able to replicate once in its permissive cells.

We previously observed that RT53 elicited signaling in all cells (reflected by cell surface PS expression) (Chapter 1, Fig 4A). Additionally, we saw caspase 2 activation also in cells surviving RT53 treatment (Chapter 1, Fig 4D). However, as hypothesized above, only cells with a particular combination of pro-apoptotic intracellular environment die in response to this signaling. Although caspase 2 is mostly known for its pro-apoptotic function, it has also been implicated in the induction of cell cycle arrest while promoting survival in live cells (Miles et al., 2017). We, therefore, cannot exclude that in the cells surviving treatment RT53 could induce factors that negatively regulate HIV-1 life cycle (for example activation of a PRR or a restriction factor). Curiously, treatment of cells with 4  $\mu$ M RT53 concentration, at which no significant increase in cell death was observed (and also no correlation between cell death and infection), already significantly inhibited Bal infection (Figure 1).



**Figure 1. RT53 displays anti-viral effect before cytotoxicity.** Median values are indicated with a line. Statistical significance was evaluated using Dunnett’s multiple comparisons test

Given our promising *in vitro* results in elimination of HIV-1 target cells with AAC-11-derived peptides, it is tempting to speculate that this approach could be useful as a potential adjunct therapy during cART or ‘shock and kill’ approaches. The peptides were already successfully used in a murine tumor xenograph model thus passing first safety barrier (Jagot-Lacoussiere et al., 2016). Despite this, several aspects of our results warrant caution when considering this therapy in monkeys or humans. First, RT53 induced cytotoxicity not only of CD4+ T cells but also of other lymphocyte populations (CD8+ T cells and B cells). This suggests that common factors responsible for the maintenance/survival of the lymphocyte lineage may be implicated in the response to RT53. This also indicates that AAC-11-derived peptides may trigger adverse effects due to their targeting of non-CD4+ T cells *in vivo*. Additionally, the

precise molecular mechanism of peptide action and associated cell death still remains to be identified. It is possible that the peptides are triggering the inflammatory types of cell death (pyroptosis or necroptosis), which was not formally evaluated in this work. In this case, they could trigger strong inflammation reaction *in vivo*, which is a known co-factor for HIV propagation. Additionally, complex network of signaling *in vivo* may change the response of cells to AAC-11-derived peptides and the response of tissues to peptide-induced death signals. Therefore, the outcome of AAC-11-derived peptide *in vivo* therapy can only be evaluated through animal trials.



**ANNEX 1:**  
**CELLULAR DETERMINANTS OF HIV PERSISTENCE ON**  
**ANTIRETROVIRAL THERAPY**

Book chapter, published

# Chapter 9

## Cellular Determinants of HIV Persistence on Antiretroviral Therapy



Anastassia Mikhailova, Jose Carlos Valle-Casuso, and Asier Sáez-Cirión

**Abstract** The era of antiretroviral therapy has made HIV-1 infection a manageable chronic disease for those with access to treatment. Despite treatment, virus persists in tissue reservoirs seeded with long-lived infected cells that are resistant to cell death and immune recognition. Which cells contribute to this reservoir and which factors determine their persistence are central questions that need to be answered to achieve viral eradication. In this chapter, we describe how cell susceptibility to infection, resistance to cell death, and immune-mediated killing as well as natural cell life span and turnover potential are central components that allow persistence of different lymphoid and myeloid cell subsets that were recently identified as key players in harboring latent and actively replicating virus. The relative contribution of these subsets to persistence of viral reservoir is described, and the open questions are highlighted.

**Keywords** HIV reservoirs · CD4+ T-cell subsets · Macrophages · Dendritic cells · HIV susceptibility · Cell survival · Turnover potential

### 9.1 Introduction

Antiretroviral therapy (ART) has achieved impressive success in preventing progression of HIV-1 infection. However, ART is lifelong and is not a cure. HIV-1 persists in viral reservoirs (Barton et al. 2016) that lead to viral rebound if ART is discontinued. A better understanding of HIV reservoirs is fundamental in the search for an HIV cure (Deeks et al. 2016; Passaes and Saez-Cirion 2014). HIV-1 persistence under ART may be due to (1) low-level viral replication, in particular in tissues where concentration of antiretrovirals (ARVs) may not always reach optimal levels (Fletcher et al. 2014; Lorenzo-Redondo et al. 2016; Tobin et al. 2005), and (2)

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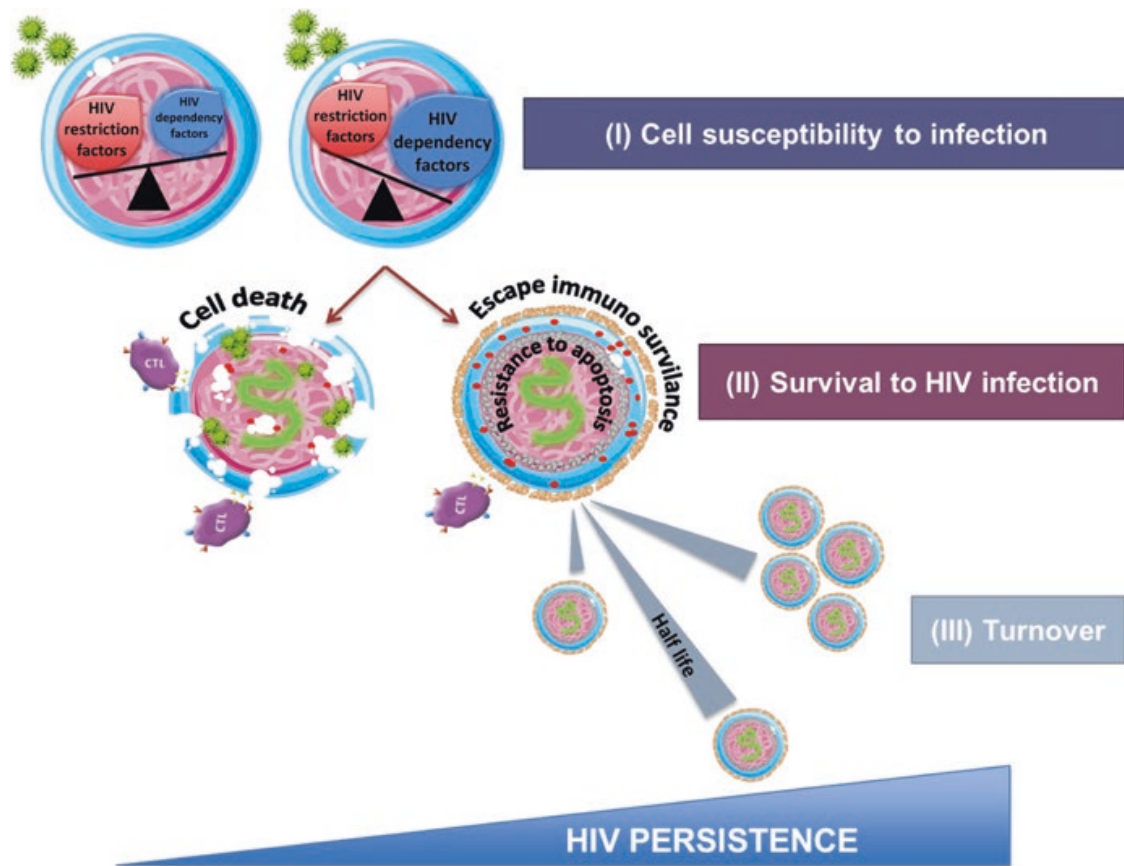
the long half-life and self-renewal of latently infected cells (Chomont et al. 2009). Although it is likely that HIV-1 persistence is a consequence of the combination of these two processes, we will focus here on intrinsic cell properties that determine the maintenance of infected cells.

HIV-1 infects cells from the myeloid and T-cell lineages, but not all cell populations contribute equally to HIV persistence (Barton et al. 2016). HIV persistence on ART is largely secondary to infected CD4<sup>+</sup> T cells (Chomont et al. 2009; Chun et al. 1998). Macrophages and dendritic cells play a critical role as early targets for HIV infection and as vehicles for HIV-1 dissemination throughout the body, but their role in long-term persistence is still unclear (Abbas et al. 2015; Coleman and Wu 2009). There are multiple subsets of CD4<sup>+</sup> T cells that follow a gradient of maturation stages including naïve (TNA), stem cell memory (TSCM), central memory (TCM), transitional memory (TTM), effector memory (TEM), and terminally differentiated (TTD) cells (Mahnke et al. 2013). In HIV-infected individuals on ART, HIV DNA is most frequently detected in memory CD4<sup>+</sup> T cells and in particular TCM and TTM (Chomont et al. 2009). However, the relative contribution of these subpopulations to the HIV reservoir may vary depending on whether the treatment was initiated in primary or chronic infection, and after long periods of ART, there is a greater contribution of very long-lived infected cells such as TSCM (Ananworanich et al. 2015; Buzon et al. 2014; Jaafoura et al. 2014; Cheret et al. 2015).

The establishment and maintenance of HIV-1 reservoirs is a multifaceted process that depends (1) on the relative cell susceptibility to HIV infection, (2) the capacity of the infected cell to resist HIV-induced apoptosis and escape immune surveillance, and (3) the infected cell's life span and turnover potential (Fig. 9.1). All these processes are determined by each cell type's program and regulated by tissue location, activation, and differentiation state of the cells in response to environmental conditions and stress signals. This chapter analyzes each of these processes with respect to how they contribute to HIV persistence on ART.

## 9.2 Being a Good or a Bad Host to the Virus

HIV-1 cell tropism is determined by the expression on the cell surface of the main HIV-1 receptor CD4 and at least one additional co-receptor, mainly CCR5 or CXCR4 (Wilén et al. 2012). CD4<sup>+</sup> T cells, monocytes/macrophages, and dendritic cells are the major targets of HIV-1 (Kandathil et al. 2016; Kumar et al. 2014). HIV-1 replicates preferentially in activated CD4<sup>+</sup> T cells and less efficiently in macrophages and immature dendritic cells, while resting CD4<sup>+</sup> T cells, monocytes, and mature dendritic cells are relatively resistant to HIV-1 infection (Steinman et al. 2003; Wu and KewalRamani 2006; Izquierdo-Useros et al. 2010; Descours et al. 2012; Diamond et al. 2004). These differences are mostly explained by the relative abundance of cellular factors that participate in the virus life cycle, either facilitating or interrupting viral replication. Several studies have identified hundreds of cellular



**Fig. 9.1 Cellular determinants for the establishment and persistence of HIV on ART.** HIV persistence is first determined by the susceptibility of different cells to infection (*i*), which is regulated by the balance of HIV host dependency factors and viral restriction factors present in the cells. In order to persist, infected cells need to resist apoptotic signals induced by viral infection and avoid immune surveillance (*ii*). These resistant infected cells will persist for variable periods of time depending on their specific life span and capacity to proliferate without enhancing HIV-dependent cell death signals (*iii*)

factors potentially required for HIV-1 to complete each step of its replication cycle, called HIV dependency factors (HDF) (Brass et al. 2008; König et al. 2008; Zhou et al. 2008; Chinn et al. 2010; Cleret-Buhot et al. 2015).

The expression of chemokine receptors varies with T-cell differentiation, impacting the susceptibility of the cells to HIV-1 (Sallusto et al. 1998; Veazey et al. 2000). Among CD4+ T helper lineages, Th2 cells are relatively resistant to HIV-1 infection, in particular to CCR5-using (R5) viruses (due to low CCR5 expression) (Gosselin et al. 2010; Sun et al. 2015) although these cells might be targeted by CXCR4-using (X4) viruses late in infection (Maggi et al. 1994). Th1 cells are susceptible to both R5 and X4 viruses but to a lower extent than Th1/Th17 or Th17 cells that express CCR6 (El Hed et al. 2010; Gosselin et al. 2010; Sun et al. 2015). The enhanced susceptibility of CCR6+ CD4+ T cells to HIV-1 infection was linked to an enhanced expression of HDF in these cells (Cleret-Buhot et al. 2015).

In addition to HIV entry receptors, some of the best known HDF include cyclophilin, which binds to HIV-1 capsid and facilitates decapsidation/reverse

transcription through an unknown mechanism (De Iaco and Luban 2014); cytoskeleton, which is required for intracellular trafficking of the virus (Menager and Littman 2016; Stolp and Fackler 2011); lens epithelium-derived growth factor (EDGF)/p75, which interacts with integrase and is responsible for the tethering and selective integration of HIV into active transcription units of the chromatin and possibly also in the regulation of HIV latency (Gerard et al. 2015; Engelman and Cherepanov 2008); positive transcription elongation factor (P-TEFb, composed of cyclin-dependent kinase 9 (CDK9) and of cyclin T1 or T2 (CycT1/T2)) and nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF $\kappa$ b) and nuclear factor of activated T cells (NAFT) transcription factors, which are required for HIV-1 transcription (Karn and Stoltzfus 2012); and endosomal sorting complex required for transport (ESCRT), which participates in HIV-1 budding (Usami et al. 2009).

While relying on numerous cellular factors, HIV-1 needs to overcome restriction factors that have evolved as part of our innate immune response to counteract infections (Arhel and Kirchhoff 2010). Although several cellular factors have been suggested to potentially inhibit HIV-1 infection (Liu et al. 2011), to date only a handful of restriction factors have been clearly validated. Serine incorporators 3 and 5 (SERINC3 and SERINC5) interfere with the delivery of viral particles to target cells (Rosa et al. 2015; Usami et al. 2015); apolipoprotein B mRNA editing enzyme catalytic subunit 3G (APOBEC3G), tripartite motif containing 5 (TRIM5 $\alpha$ ), and SAM domain- and HD domain-containing protein 1 (SAMHD1) impair reverse transcription (Santa-Marta et al. 2013; Simon et al. 2015); MX dynamin-like GTPase 2 (Mx2) hinders nuclear accumulation and integration of proviral DNA into the host chromatin (Goujon et al. 2013); bone marrow stromal cell antigen 2 (BST-2) retains newly produced viral particles at the surface of infected cells (Perez-Caballero et al. 2009). The strong expression of HDF present in immune cells suggests that HIV-1 needed to evolve to replicate in these cells, which contain critical factors for its replication cycle, and adapted to circumvent the action of restriction factors (Brass et al. 2008; Cleret-Buhot et al. 2015). Thus, the susceptibility of host cells to HIV-1 infection is largely dictated by the availability of HDF rather than the expression of restriction factors, with the exception of SAMHD1.

SAMHD1 possesses dNTPase and nuclease activities and can potentially interfere with HIV-1 reverse transcription by reducing the pool of intracellular deoxy-nucleoside triphosphate (dNTP) and by degrading incoming viral nucleic acids (Beloglazova et al. 2013; Laguette et al. 2011; Lahouassa et al. 2012), although the relative contribution of each of these activities is still unclear. This may vary as a function of cell type and cell cycle. SAMHD1 efficiently blocks HIV-1 infection in quiescent CD4<sup>+</sup> T cells and monocytes and strongly decreases HIV-1 dynamics in differentiated myeloid cells, but it is inefficient in cycling CD4<sup>+</sup> T cells (Laguette et al. 2011; Cribier et al. 2013).

The differences in the antiviral activity of SAMHD1 between these cell types are not related to its relative expression, but SAMHD1 antiviral activity is ablated by the phosphorylation of its threonine 592 (T592) residue (Cribier et al. 2013; White et al. 2013). Phosphorylation of SAMHD1 is regulated by cyclin-dependent kinases (CDK) 1/2, which coordinate T-cell division and differentiation in response to

antigen recognition (Wells and Morawski 2014). A recent report shows that tissue resident macrophages susceptible to HIV-1 infection such as microglial cells, which are responsible for HIV-1 persistence and compartmentalization in the brain (Schnell et al. 2011), are in a G1-like status and express high levels of phosphorylated (inactive) SAMHD1 (Mlcochova et al. 2017).

The influence of the cell cycle on HIV-1 replication is also well known (Goh et al. 1998). P21<sup>cip1/waf1</sup>, a CDK inhibitor which regulates cell cycle arrest and is involved in monocyte differentiation (Asada et al. 1999; Xiong et al. 1993), is a potent inhibitor of HIV-1 infection (Bergamaschi et al. 2009). On the one hand, p21 controls the de novo synthesis of dNTPs by regulating the expression of the main enzymes involved in this process (Allouch et al. 2013; Valle-Casuso et al. 2017). Through its CDK inhibitor activity, p21 also controls the phosphorylation state of SAMHD1 (Allouch et al. 2014; Pauls et al. 2014b). p21 has also been shown to interfere with HIV-1 replication in hematopoietic stem cells (Zhang et al. 2007) and with CDK9-dependent transcription of HIV-1 in CD4+ T cells (Chen et al. 2011).

The differentiation state of the cells also influences the relative capacity of HIV-1 to replicate. HIV-1 replicates less well in naïve CD4+ T cells and monocytes than in memory CD4+ T cells and macrophages, and this is not only related to differential expression of HIV-1 co-receptors. Successful HIV infection requires stable integration of viral cDNA into the host cell genome. HIV integration occurs preferentially within transcription units of transcriptionally active genes (Han et al. 2004; Schroder et al. 2002). However, several mechanisms including epigenetic gene silencing, transcription gene silencing, and posttranscriptional gene silencing have been described to explain the establishment and maintenance of latency in target cells (reviewed in Van Lint et al. (2013)). Although these mechanisms are described in detail elsewhere in this book, it is interesting to note here that HIV gene expression is heavily dependent on the presence of several transcription factors, such as NFAT and NFκB. These factors are critical regulators of T-cell activation and differentiation, and their expression is necessary for rapid production of cytokines or effector molecules (Macian 2005; Oh and Ghosh 2013). Accordingly, NFAT and NFκB are expressed at very low levels in naïve and resting T cells and strongly expressed in activated and differentiated T cells.

In vitro studies suggest that latency can be established in both resting and activated CD4+ T cells (Chavez et al. 2015). However, it is reasonable to think that HIV-1 latency per integration event may be achieved more frequently in less-differentiated CD4+ T cells subsets than in effector cells, but this remains to be proven. The mammal target of rapamycin (mTOR) is a pivotal regulator of cell differentiation, cell cycle, proliferation, and survival (Chi 2012). mTOR directly regulates many HDF (e.g., cytoskeleton, NFAT) and it has been shown to control HIV-1 latency (Besnard et al. 2016). mTOR also regulates the metabolic activity of the cells (Powell and Delgoffe 2010), and expression of the glucose transporter Glut1 is required for HIV-1 replication (Loisel-Meyer et al. 2012). Thus, it is likely that mTOR has an important part in the regulation of HIV-1 infection in different cell subsets.



Much less evidence is available about the establishment of HIV latency in infected macrophages. As in CD4+ T cells, in macrophages HIV preferentially integrates into the transcriptionally active region of the chromatin (Kumar et al. 2014). However, it is not clear whether the mechanisms driving latency in CD4+ T cells are similar in macrophages. Latency can be established in macrophages in vitro (Brown et al. 2006), and HIV transcription is regulated in response to external signals and macrophage activation (Saez-Cirion et al. 2006). Moreover, the transcription factor B-cell CLL/lymphoma 11B (BCL11B/CTIP2), which is involved in multiple cellular processes including cell proliferation and survival, has been shown to repress HIV gene transcription in microglial cells by inhibiting the elongation factor P-TEFb and by inducing a compact, transcriptionally inactive, heterochromatic environment at the HIV promoter (Le Douce et al. 2010).

Although HIV-1 has evolved mechanisms to avoid the action of restriction factors such as APOBEC3G, this factor is expressed at very high levels in the cells that are more susceptible to HIV-1 infection, and this may come at a cost for the virus in terms of replication capacity in more differentiated cell subsets (Vetter et al. 2009). Finally, some cells, such as dendritic cells, are poorly or not susceptible to HIV-1 replication but can internalize free virions in non-cytolytic vesicles and transfer them at high concentrations to CD4 T cells upon interaction (Manches et al. 2014). Along these lines, follicular DCs have been shown to trap and retain infective HIV-1 particles for extended periods of time (Heesters et al. 2015).

## 9.3 Dodging Cell Death Upon Infection

### 9.3.1 Apoptosis

Although apoptosis is a major mechanism of defense against infection, viruses have evolved means to influence the balance of death and survival of the host cell in order to promote efficient virus replication and persistence of infection. Progressive CD4+ T-cell loss is a defining characteristic of uncontrolled HIV-1 infection. HIV-1 can provoke direct cytotoxicity on target cells. However, it is now well accepted that decline of CD4+ T cells in vivo is not solely due to direct viral cytotoxicity but to a multifactorial process that also includes apoptosis of “bystander” cells (Doitsh et al. 2010; Doitsh et al. 2014; Finkel et al. 1995) and killing of productively infected cells by immune effectors. Yet persistence of HIV-1 infected cells for long periods of time requires avoiding all these forms of cell death. Studies performed in vitro and ex vivo have shown the contribution of many different apoptotic molecules in CD4+ T cells and other HIV-1 susceptible cell subsets. The exact molecular mechanisms of HIV-1-induced cytotoxic or anti-apoptotic effects on infected cells that lead to long-lived HIV persistence are still not well understood.

Viral proteins such as tat (Li et al. 1995; Westendorp et al. 1995), env (Cicala et al. 2000), vpr (Muthumani et al. 2002), and nef (Muthumani et al. 2005; Xu et al. 1999) have been shown to have a pro-apoptotic effect in vitro. However, the action

of these proteins at physiological concentrations and in a complex immunological setting remains unclear. Moreover, the regulation of apoptosis depends on the interaction of viral factors with cell pathways, and the equilibrium between pro-apoptotic and anti-apoptotic signals may vary as a function of the stage of viral replication, nature, and state of the target/bystander cell and external signals. Nef has been shown to prevent apoptosis in productively infected cells upon Fas and tumor necrosis factor alpha (TNFalpha) ligation (Ohnismus et al. 1997) by inhibiting Fas signaling (Geleziunas et al. 2001). Nef was also shown to inactivate pro-apoptotic BCL2-associated agonist of cell death (Bad) protein, hence rendering infected T cells more resistant to apoptosis (Wolf et al. 2001).

On the other hand, myeloid lineage cells including monocytes and macrophages appear less sensitive to the cytopathic effect of HIV replication than T cells, suggesting that intrinsic properties of myeloid cells may render them selectively resistant to HIV-induced apoptosis (Kumar et al. 2014; Le Douce et al. 2010). Along these lines, telomerase activity increases in macrophages upon infection, rendering them more resistant to DNA damage or oxidative stress (Ojeda et al. 2014). Macrophages were observed to be more apoptosis resistant at least in part due to env (Swingler et al. 2007)- and nef (Olivetta and Federico 2006)-dependent alterations in apoptotic pathways.

Following ART, the contribution of direct viral cytotoxicity to cell death is likely minimal. Latently infected cells remain largely ignored by HIV-specific T cells due to the lack of expression of viral proteins, which favors persistence. However, infected CD4+ T cells have been reported to undergo integration-dependent cell death, linked to the recruitment of DNA-dependent protein kinase (DNA-PK) (Cooper et al. 2013). It is unlikely that latency is an “all or nothing” phenomenon, and it is possible that episodes of viral reactivation occur episodically in vivo even in the presence of effective cART (Nettles et al. 2005; Graf et al. 2013a). Recent studies have shown that reactivation of latently infected cells in ART-suppressed HIV-infected individuals with histone deacetylases (HDAC) inhibitors or other latency reversal agents was insufficient to promote cell death ex vivo or to decrease the frequency of integrated viral DNA or infectious units in vivo (Lehrman et al. 2005; Rasmussen and Lewin 2016; Rasmussen et al. 2014; Routy et al. 2012; Shan et al. 2012). Moreover, cells carrying latent replication competent viruses might be particularly resilient to CD8+ T cell-mediated killing even after viral reactivation (Huang and Jones 2017). Therefore, long-term HIV-1 persistence may be caused by cells that are particularly resistant to cell death, either due to their intrinsic properties or because of an anti-apoptotic state, supported by HIV factors.

Different studies using in vitro models of HIV-1 latency have described that establishment of latent HIV-1 infection is accompanied by the induction of anti-apoptotic proteins (e.g., B-cell CLL/lymphoma 2 (BCL2), cellular FLICE-like inhibitory protein (cFLIP), myeloid cell leukemia sequence 1 (Mcl-1) (Aillet et al. 1998; Berro et al. 2007; Tan et al. 2013) or the downregulation of pro-apoptotic proteins (e.g., BCL2-associated X protein (BAX), Fas-associating death domain-containing protein (FADD)) (Wang et al. 2011; Badley et al. 2013). Interestingly, in vivo, cells that support virus persistence such as TCM (Olvera-García et al. 2016)



and monocytes (Giri et al. 2009) had an anti-apoptotic gene signature in HIV-infected individuals compared to noninfected controls.

Death of nonproductively infected nonactivated T cells that do not express viral antigen has been reported to result from the detection of viral reverse transcription products by DNA sensor interferon gamma inducible protein 16 (IFI16), which leads to caspase-1 activation, thereby triggering pyroptosis (Doitsh et al. 2010; Monroe et al. 2014). However, the contribution of this form of cell death is probably limited once ART is initiated (Cai et al. 2016).

Apoptosis of bystander cells has also been linked to persistent immune activation seen in chronic infection via signaling by TNF family members (TNF-related apoptosis-inducing ligand (TRAIL), Fas ligand (FasL), and TNFalpha) (Herbeuval et al. 2005, 2006; Katsikis et al. 1995; Sloand et al. 1997). Death signals delivered via Fas ligation (Katsikis et al. 1995; Sloand et al. 1997) were shown to have an important contribution to bystander cell depletion in HIV-infected individuals (Badley et al. 1997; Badley et al. 1996, 1998). However, these signals were observed to be counteracted, at least in part, by viral proteins expressed by infected cells. For example, Env was observed to induce resistance to TRAIL-induced apoptosis in macrophages (Swingler et al. 2007).

Overall, it seems that the activation status of cells plays a role in the susceptibility to cell death during HIV infection, which is also reflected by differential loss of cell populations from different tissues, where more or less activated cell phenotypes are found. For example, in the lymph nodes of HIV-infected individuals, the degree of apoptosis has been correlated with virus- and microbial-driven immune activation observed in infection and not the viral load (Muro-Cacho et al. 1995). It has been further shown that highly activated effector memory CD4<sup>+</sup> T cells are depleted faster and first from gut mucosal sites (Grossman et al. 2006) and naïve T cells displaying a resting phenotype are resistant to depletion in lymphoid tissues (Veazey et al. 2000). Additionally, blood-derived CD4<sup>+</sup> T cells that display a deeper resting state than lymphoid tissue-derived cells are more resistant to pyroptosis despite carrying viral genetic material (Muñoz-Arias et al. 2015). Thus, infected naïve, central memory, and stem cell-like memory T cells displaying a less activated phenotype could be less prone to these mechanisms of induced cell death than highly activated effector memory T cells. These differences could contribute to shaping where HIV persists, even before treatment initiation.

The relevance of these mechanisms during ART is unknown. ART undoubtedly halts loss of CD4<sup>+</sup> T cells, but some level of abnormal chronic inflammation persists (Paiardini and Muller-Trutwin 2013), and it is likely that this may contribute to selective elimination during treatment of cells that have the highest susceptibility to apoptosis. The susceptibility of infected cells to cell death may also vary in tissues depending on the cytokine milieu. For example, interleukin (IL)-7 protected resting CD4<sup>+</sup> T cells from death during *in vitro* HIV infection (Trinité et al. 2016), whereas IL-12 protected while IL-10 augmented Fas-mediated cell death of CD4 T cells from HIV-infected individuals (Estaquier et al. 1995).

### 9.3.2 Immune Clearance

HIV-1-specific cytotoxic CD8+ T cells and natural killer (NK) cells are able to eliminate infected cells, and these responses have been linked to protection against HIV transmission or natural control of infection (Betts et al. 2006; Graf et al. 2013b; Jennes et al. 2006; Martin et al. 2002; Ravet et al. 2007; Sáez-Cirión et al. 2007; Sáez-Cirión et al. 2009). However, during progressive infection, selection in vivo of viral variants that escape this immune pressure occurs progressively diminishing the capacity of these cells to counteract infection (Alter et al. 2011; Borrow et al. 1997; Deng et al. 2015; Mailliard et al. 2013). In elite controllers, HIV-1 persists for many years even in the presence of highly efficient CD8+ T-cell responses (Noel et al. 2016), suggesting that persistent infected cells are able to avoid immune surveillance. Latently infected resting CD4+ T cells that do not actively express viral epitopes escape immune surveillance although transient expression of viral antigens may trigger their killing (Graf et al. 2013a). Viral proteins such as Nef (Cohen et al. 1999) and Vpu (Apps et al. 2016) downregulate major histocompatibility complex (MHC) class I and may contribute to protect infected CD4+ cells from the CD8+ T-cell response (Collins et al. 1998; Xu et al. 1997) (although this could make these targets susceptible to NK mediated killing (Cohen et al. 1999)).

Infected macrophages might be more resistant than CD4+ T cells to killing by CD8+ T cells in vitro, independently of nef (Rainho et al. 2015; Vojnov et al. 2012). Infected macrophage can be eliminated by HIV-specific cytotoxic CD4+ T cells (Sacha et al. 2009), which have been found to increase during acute infection (Soghoian et al. 2012) and in elite controllers (Johnson et al. 2015). In addition, in macrophages, HIV-1 particle assembly in intracellular virus-containing compartments (VCCs) (Jouve et al. 2007; Welsch et al. 2011) may provide protection from immune recognition (Tan and Sattentau 2013).

In addition to escape mutations and latency, effective immune responses are also curtailed by the physical separation of effector cells from their targets residing in tissue sanctuaries. The central nervous system (CNS) has long been considered an “immune-privileged” site where infected macrophages, astrocytes, and microglial cells are relatively inaccessible to antiviral immune responses and variably accessible to ART (Letendre et al. 2008; Joseph et al. 2015), thus constituting an important viral reservoir.

Cerebrospinal fluid (CSF) from noninfected individuals contains CD4+ T cells with an activated central memory phenotype (Kivisäkk et al. 2003), which are a preferential target of HIV-1. Compared to the blood, the ratio of CD8/CD4 T cells is much lower in the CNS, and it has been suggested that antigen-specific CD8+ T cells found in the CNS do not provide durable immune surveillance in the absence of antigen (Young et al. 2011). Resident memory CD8+ T cells can be found in the CNS in the context of viral infection (Wakim et al. 2010), and it is now recognized that functional HIV-1-specific CD8+ T cells infiltrate CNS during acute (Kessing et al. 2017) and chronic (Ganesh et al. 2016) HIV infection. Remarkably, CD8+ T-cell responses in the CNS are detected in elite controllers with undetectable viral

load in the CSF and blood (Sadagopal et al. 2008) and during ART (Lescure et al. 2013; Miller et al. 2004) and contribute to the control of infection in the CNS (Marcondes et al. 2015).

Several reports have shown accumulation of virus bearing Tfh cells in germinal centers of lymphoid follicles (Banga et al. 2016; Perreau et al. 2013) where CD8+ T cells are found in low frequencies as compared to T-cell zones (Connick et al. 2007; Folkvord et al. 2005; Fukazawa et al. 2015; Hong et al. 2012). However, recently identified follicular cytotoxic T cells (Tfc) were shown to enter B-cell follicles of HIV-infected individuals and to have cytotoxic potential (Leong et al. 2016; Petrovas et al. 2017). Cytotoxic CD8+ T cells displaying viral target lysis are also detected in the lamina propria (Murphey-Corb et al. 1999) as well as vaginal epithelium and submucosa (Lohman et al. 1995) of SIV-infected rhesus macaques. Activated CD8+ T cells with cytotoxic potential were also located in adipose tissue (which carried infected CD4+ T cells and macrophages) of SIV-infected monkeys (Damouche et al. 2015). It is, therefore, probable that HIV-specific CD8+ T-cell responses in tissue contribute to the elimination of infected cells in nonlymphoid tissues and even the CNS, although more studies will be needed to define their characteristics.

## 9.4 Endurance and Renovation

The number of cells containing HIV DNA decreases sharply during the first months following initiation of ART (Finzi and Siliciano 1998; Avettand-Fenoel et al. 2016). A steady state appears to be reached by 2 years on ART, although DNA decay continues for longer periods of time when ART is initiated during primary infection (Avettand-Fenoel et al. 2016). Modeling the dynamics of decline in HIV DNA showed that this decay occurs in several phases that have been attributed to the sequential loss of infected cells of varying half-life (Finzi and Siliciano 1998; Perelson 2002). In the context of ART efficiently blocking systemic HIV replication, cells with active viral replication are expected to be eliminated within a few days due to cytopathic effects or immune clearance (see above). However, some cells like macrophages can produce infectious viral particles for long periods of time without being killed and resting CD4+ T cells carrying latent provirus persist despite multiple decades of treatment.

The maintenance of infected CD4+ T cells under ART is driven by survival of long-lived cells and homeostatic proliferation (Chomont et al. 2009). The life span of quiescent CD4+ T cell progressively decreases with differentiation. Naïve CD4+ T cells are much longer lived than memory cells, and early differentiated memory CD4+ T cells have a longer half-life than terminally differentiated cells. TNA, TSCM, and TCM upregulate genes associated with survival and are less prone to undergo apoptosis, at least in vitro (Lugli et al. 2013; Mahnke et al. 2013). It has been estimated that one TNA has a half-life of 1 to several years, a TCM a few months to 1 year, while a TEM would only be a few weeks (Macallan et al. 2004;

Vrisekoop et al. 2008). TSCM have the highest survival capacity among memory T cells in the absence of cognate antigen (Lugli et al. 2013; Gattinoni et al. 2011). However, these estimations, largely based on the *in vivo* analysis of incorporation of deuterated glucose or water on CD4+ T cells, are limited by the lack of resolution on cells that migrate to the tissues. The recently described resident memory T cells are programmed to persist locally and not recirculate even in the absence of antigen (Clark 2015). The role of these cells in the context of HIV-1 infection has not been clarified yet but their potential contribution to the persistence of HIV on ART deserves analysis.

The contribution of infected macrophages to HIV persistence on ART is debated (DiNapoli et al. 2017). Resting CD4+ T cells constitute the bulk of persisting infected cells, but the potential implication of infected macrophages as source of rebounding virus if treatment is interrupted should not be overlooked (Crowe et al. 2003). Although it is accepted that viral decay in monocytes/macrophages is slower than in activated CD4+ T cells, it is often assumed that the half-life of infected monocytes/macrophages is shorter than that of quiescent CD4+ T cells (Finzi and Siliciano 1998; Stevenson 2003; Van Lint et al. 2013). However, the life span of macrophages, as for CD4+ T cells, also varies greatly. Depending on their tissue location, macrophages can live from a few months to several years. Alveolar macrophages, which can be infected by HIV (Jambo et al. 2014), have been found to persist for over 3 years in analyses performed after lung transplant (Eguiluz-Gracia et al. 2016; Nayak et al. 2016), and microglial cells persist for years in the CNS (Tay et al. 2017). Moreover, macrophages may be better prepared than CD4+ T cells to resist apoptosis under conditions of metabolic stress (Carter and Ehrlich 2008; Jones and Power 2006; McNelis and Olefsky 2014).

It was previously assumed that activation of HIV-infected CD4+ T cells driving them to proliferation would reverse viral latency and decrease the half-life of cells carrying replication competent virus. However, recent phylogenetic studies using ultra deep whole genome sequencing have shown the presence of proviruses with identical sequences in clonally expanded infected CD4+ T cells (Chomont et al. 2009; Cohn et al. 2015; Maldarelli et al. 2014; Simonetti et al. 2016; Wagner et al. 2014; Boritz et al. 2016). Moreover, it is now clear that these expanded infected CD4+ T cells can also harbor intact proviruses able to spread infection (Hosmane et al. 2017; Simonetti et al. 2016). Several reports have shown that proliferation of infected CD4+ T cells could be at least partially driven by the selective integration of HIV-1 into genes that have been associated with cell growth, division, and cancer (Maldarelli et al. 2014; Wagner et al. 2014).

In addition, CD4+ T cells can divide in response to antigenic stimulation or to homeostatic signaling to balance cell numbers although capacity of self-renewal is lost with progressive differentiation of memory CD4+ T cells (Berard and Tough 2002; Mahnke et al. 2013). Antigenic stimulation through the T-cell receptor entails the activation of the cell and triggers cell differentiation. However, naïve and early differentiated cells require a higher signaling threshold and prolonged contact with antigen-presenting cells, and they also depend more on co-stimulatory signals than more differentiated cells to respond to antigens. Thus, low levels of antigen during

treated infection might provide a suboptimal signal allowing some degree of activation of these cells. It is however unknown whether, *in vivo*, some transiently activated cells might escape cell death despite some degree of viral production to later regain a quiescent state. In contrast, *in vitro* studies have confirmed that infected CD4<sup>+</sup> T cells can undergo homeostatic proliferation without significant viral production or cell death (Bosque et al. 2011).

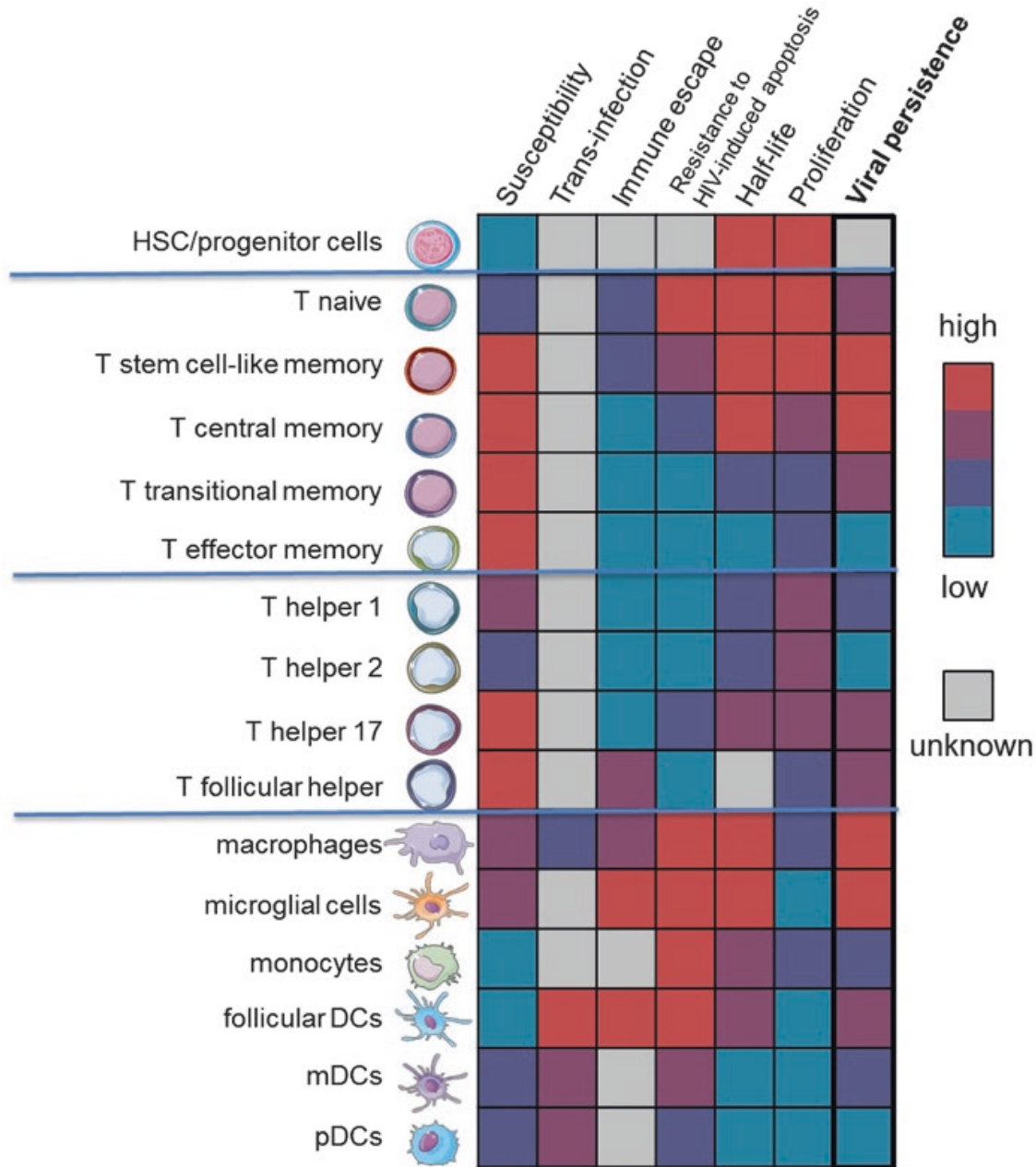
Homeostatic proliferation is governed by members of the common gamma chain family of cytokines in the absence of antigenic stimulation (Boyman et al. 2007; Seddon et al. 2003; Surh and Sprent 2008). In particular, IL-7 plays a central role in CD4<sup>+</sup> T-cell homeostasis and survival. In the case of naïve CD4<sup>+</sup> T cells, IL-7 signaling and contact with self-MHC-peptides complexes promotes cell survival without inducing proliferation. In contrast, IL-7 signaling can promote proliferation of memory CD4<sup>+</sup> T cells independently of TCR activation. Responsiveness to IL-7 is not equal among all CD4<sup>+</sup> T-cell memory subsets. TSCM and TCM express high levels of the IL-7 receptor (CD127) and have strong proliferative potential, while TEM express lower levels of CD127 and have a limited proliferative potential (Mahnke et al. 2013). Other common gamma chain cytokines (such as IL-2 or IL-15) also influence the survival and turnover of T cells in their inflammatory environment (Pennock et al. 2013). Overall, once established, memory CD4<sup>+</sup> T cells can persist for decades in the absence of antigen (Hammarlund et al. 2003).

Macrophages and dendritic cells are terminally differentiated cell populations that cannot be propagated *in vitro*. However, macrophage subsets that are susceptible to HIV-1 infection are not in a quiescent state and share some characteristics of cycling cells (Badia et al. 2016; Mlcochova et al. 2017; Pauls et al. 2014a). Although infected macrophages did not show evidence of division *in vitro*, it is now clear that *in vivo* some macrophages can proliferate locally in tissues in response to inflammatory signals (Jenkins et al. 2011; Robbins et al. 2013; Zamarron et al. 2016). Thus, the possible persistence of some infected macrophages through cell division cannot be discarded.

## 9.5 Conclusion

The persistence of HIV-1-infected cells on ART depends on a combination of cell intrinsic characteristics including the susceptibility of cells to infection and their capacity to survive and proliferate (Fig. 9.2). However, it is also influenced by the responsiveness of these cells to external signals (such as inflammatory cytokines or contact with antigen-presenting cells) or their localization and capacity to circulate. For instance, HIV-specific CD4<sup>+</sup> T cells are preferentially infected by HIV during treatment interruption when compared to other antigen-specific memory CD4<sup>+</sup> T cells, and this is likely due to the selective localization of activated HIV-specific cells to the sites of viral replication (Douek et al. 2002). HIV-1 can thus reside in multiple cell subsets in multiple tissues, while the main mechanisms for persistence are diverse. It is of the utmost importance to define which specific infected cell





**Fig. 9.2 Heatmap showing multiple features of different cell subsets that lead to HIV persistence on ART.** Color codes were inferred on the basis of the literature discussed in this chapter. Among T-cell subsets, naive, central memory, and stem cell-like memory T cells have a major contribution to long-term HIV persistence on ART, while effector memory and terminally differentiated helper T-cell subsets are depleted first during infection and have limited proliferative potential. Myeloid cells, despite their relatively low susceptibility to infection, are now increasingly recognized as important contributors to HIV persistence on ART in both lymphoid and non-lymphoid tissues due to their long half-life and resistance to apoptosis and immune-mediated killing. Although not shown here, tissue localization and activation status influence various parameters of persistence, with actively infected cells being more susceptible to cell death, thus reducing their contribution to long-term persistence

subset leads to viral rebound off ART and to determine if some of these subsets should be preferentially targeted. Irrespective, tackling HIV persistence will require multiple diverse strategies that target these difference mechanisms leading to cell survival.

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## **ANNEX 2:**

# **CELLULAR METABOLISM IS A MAJOR DETERMINANT OF HIV-1 RESERVOIR SEEDING IN CD4+ T CELLS AND OFFERS A UNIQUE OPPORTUNITY TO TACKLE INFECTION**

Valle-Casuso et al, submitted

1 **Cellular metabolism is a major determinant of HIV-1 reservoir seeding in CD4+ T cells and**  
2 **offers an opportunity to tackle infection**

3

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**ABSTRACT**

HIV persists in pools of long-lived infected cells that are not susceptible to current antiretroviral treatments. Targeting these HIV reservoirs is one of the main priorities in the HIV field. HIV reservoirs are mainly located in CD4+ T cells, but their distribution is variable in the different T cell subsets. CD4+ T cell susceptibility to HIV-1 infection varies with T cell differentiation, with naïve T cells strongly resistant to infection and effector memory cells highly susceptible. We evaluated whether the metabolic programming that supports the differentiation and function of CD4+ T cells affected the susceptibility of CD4+ T cell subsets to HIV infection. We found that differences in HIV susceptibility between naïve and more differentiated CD4+ T cell subsets were associated with the metabolic activity of the cells. We further found that HIV-1 selectively infected CD4+ T cells with high levels of oxidative phosphorylation and glycolysis, independent of their activation phenotype. Moreover, partial inhibition of glycolysis was sufficient to (i) impair productive and latent HIV-1 infection in vitro in all CD4+ T cell subsets, (ii) decrease the viability of pre-infected cells, and (iii) preclude HIV-1 reactivation in CD4+ T cells from HIV-infected individuals undergoing cART. Our results elucidate the link between cell metabolism and HIV-1 infection and identify a vulnerability that can be exploited to tackle HIV reservoirs.

## 48 INTRODUCTION

49 Current combination antiretroviral treatment (cART) efficiently blocks HIV-1 replication but does not  
50 eliminate infected cells. Replication competent HIV-1 persists for decades in cellular reservoirs that  
51 are the origin of rapid viral rebound when treatment is interrupted (Finzi et al., 1997). Identifying the  
52 factors underlying the seeding and survival of HIV-infected cells may guide the development of novel  
53 therapies against HIV and is a priority in the search for an HIV cure (Deeks et al., 2016; Passaes and  
54 Saez-Cirion, 2014). CD4+ T cells are the major target for HIV-1 infection and are thought to constitute  
55 most of the HIV-1 reservoir. However, not all CD4+ T cells contribute equally to the pool of  
56 persistently infected cells during cART. The composition of CD4+ T cells that remain infected is mainly  
57 determined by the relative susceptibility of CD4+ T cell subsets to HIV infection, their resistance to  
58 HIV-induced apoptosis and their life span and turnover potential (Barton et al., 2016). Naïve CD4+ T  
59 cells are highly resistant to HIV-1 infection, while HIV-1 susceptibility increases in more differentiated  
60 cell subsets (Roederer et al., 1997; Schnittman et al., 1990; Spina et al., 1997). Accordingly, there is a  
61 minimal contribution of naïve CD4+ T cells to the HIV reservoir during cART, which is mainly  
62 restricted to the memory cell subsets (Chomont et al., 2009). The susceptibility of CD4+ T cells to  
63 HIV-1 infection depends on the relative abundance of cell factors required by the virus to complete  
64 its replication cycle and of cellular restriction factors that counteract infection (Lever and Jeang,  
65 2011). T cell activation sharply increases the expression of HIV dependency factors and thereby cell  
66 susceptibility to HIV-1 infection (Pan et al., 2013; Stevenson et al., 1990), despite the concomitant  
67 presence of some restriction factors that the virus can most often circumvent. However,  
68 responsiveness to TCR activation (Byrne et al., 1988; Roederer et al., 1997) and susceptibility to HIV  
69 infection are not homogeneous across or within CD4+ T cell subsets. This discrepancy in infection  
70 efficacy suggests that HIV-1 has adapted to infect CD4+ T cells with a specific cellular program  
71 (Cleret-Buhot et al., 2015). The cellular processes orchestrating the optimal conditions for the  
72 establishment of HIV-1 infection remain unclear.

73



74 Numerous studies have recently demonstrated the critical role of cellular metabolism in T cell  
75 immunity (Pearce et al., 2013; Waickman and Powell, 2012). Naïve T cells circulate in a quiescent  
76 state, relying essentially on oxidative phosphorylation (OXPHOS). However, upon T cell activation and  
77 after receiving appropriate cues (costimulation, cytokines), naïve T cells undergo metabolic  
78 reprogramming, strongly increasing OXPHOS and, especially, glycolysis, to cope with the energy  
79 demands of immune function and rapid proliferation (Pearce et al., 2013). The biomass accumulation  
80 that accompanies enhanced cellular metabolism may provide viruses with the abundance of factors  
81 that are necessary for their replication. It is worth noting that several retroviruses have evolved to  
82 use metabolite transporters as cellular receptors. The glucose transporter 1 (Glut-1) is the main  
83 receptor for HTLV-1 (Manel et al., 2003); phosphate transporters Pit1 and Pit2 have been reported as  
84 surface receptors for koala retrovirus, feline leukemia virus and murine leukemia viruses (Oliveira et  
85 al., 2006; Shojima et al., 2013; Takeuchi et al., 1992; von Laer et al., 1998); and the amino acid  
86 transporters ASCT1 and ASCT2 are the receptors for the feline RD-114 endogenous retrovirus  
87 (Shimode et al., 2013). Although HIV-1 does not use metabolite transporters as its main receptors,  
88 Glut-1 expression is necessary for the postentry steps of HIV-1 replication in CD4+ T cells (Loisel-  
89 Meyer et al., 2012). Moreover, the metabolism of nucleotides, which is controlled by glycolysis  
90 through the pentose phosphate pathway (Lane and Fan, 2015), is critical for HIV-1 reverse  
91 transcription (Amie et al., 2013).

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93 In the present study, we undertook the analysis of the conditions determining the intracellular  
94 susceptibility of CD4+ T cell subsets to HIV-1 infection. In particular, we analyzed whether the  
95 metabolic program is distinct according to the differentiation of CD4+ T cell subsets and if this  
96 determines their susceptibility to HIV-1 infection. We show that cellular metabolism is a central  
97 factor driving the HIV-1 infection of CD4+ T cells and that it may be an important target for new  
98 therapies against HIV-1.

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100

101 **RESULTS**

102 **CD4+ T cell subsets have heterogeneous susceptibility to HIV-1 infection**

103 We first assessed the relative intrinsic susceptibility of primary CD4+ T cell subsets (naïve, T<sub>n</sub>; central  
104 memory, T<sub>cm</sub>; transitional memory, T<sub>tm</sub>; and effector memory, T<sub>em</sub>) to HIV-1 infection. We used  
105 single-cycle NL4.3ΔenvGFP particles pseudotyped with VSV-G envelope protein to circumvent  
106 differences in the surface expression of CCR5 across CD4+ T cell subsets. We activated CD4+ T cells  
107 with soluble anti-CD3. This 'suboptimal' activation protocol has allowed us to expose differences in  
108 the susceptibility to HIV-1 of CD4+ T cells from different individuals, in particular resistance in cells  
109 from individuals naturally controlling infection, that were masked using more potent stimulation  
110 protocols (Saez-Cirion et al., 2011). In the present study, our activation protocol allowed the  
111 enhancement of the susceptibility to HIV-1 infection without the modification of the relative  
112 proportion of the CD4+ T cell subsets (Figure 1A, B). After infection, the relative frequencies of T<sub>n</sub>,  
113 T<sub>cm</sub>, T<sub>tm</sub> and T<sub>em</sub> cells among GFP-negative (GFP-) cells was identical to that among noninfected  
114 CD4+ T cells (Figure 1B). In contrast, the composition of HIV-infected GFP-positive (GFP+) cells was  
115 different from that of noninfected cells, with a significant exclusion of T<sub>n</sub> cells and strong enrichment  
116 of T<sub>em</sub> cells. T<sub>cm</sub> cells were also slightly underrepresented, and T<sub>tm</sub> cells were overrepresented  
117 among GFP+ CD4+ T cells when compared to the control condition (Figure 1B). These results  
118 suggested different susceptibilities to HIV-1 infection of CD4+ T cell subsets, with T<sub>em</sub> cells being the  
119 most susceptible, followed by T<sub>tm</sub> and T<sub>cm</sub> cells, and with T<sub>n</sub> cells being strongly resistant to  
120 infection.

121

122 To study if these differences were related to the inherent program of each CD4+ T cell subset, we  
123 isolated quiescent (CD25-, CD69-, HLA-DR-) CD4+ T<sub>n</sub>, T<sub>cm</sub>, T<sub>tm</sub> and T<sub>em</sub> cells (n=6 donors, Figure S1)  
124 using flow-based cell sorting, and we analyzed their susceptibility to HIV-1 with or without anti-CD3  
125 activation. Activation enhanced the susceptibility of all CD4+ T cell subsets to HIV-1 infection (Figure  
126 1C). However, this effect was variable according to the subset. There was a tendency for T<sub>em</sub> cells to

127 be more susceptible than other subsets ( $p=0.06$ ) in the absence of activation, and this difference  
128 became more pronounced after three ( $p=0.0004$ , all comparisons) or five days of activation ( $p=0.012$ ,  
129 Tem vs Tn and Ttm and Tcm vs Tn and Ttm). Overall, our results recapitulated previous observations  
130 showing an inherent hierarchy in the susceptibility of CD4+ T cell subsets to HIV-1 infection (Buzon et  
131 al., 2014; Tabler et al., 2014).

132

### 133 **Levels of HIV infection are related to the molecular program of CD4+ T cell subsets**

134 To determine if a molecular program was associated with the susceptibility of CD4+ T cell subsets to  
135 HIV infection, we analyzed the expression of a panel of 96 genes (related to T cell activation, survival,  
136 differentiation and function as well as known viral restriction or HIV facilitating factors, Table S1) in  
137 each CD4+ T cell subpopulation at the time of infection. Nonactivated CD4+ T cell subsets showed  
138 distinct transcriptional profiles that were further enhanced after activation (e.g., 34 genes and 49  
139 genes differently expressed between CD4+ T cell subsets without activation and after 3 days of anti-  
140 CD3 treatment, respectively, Figure 2A). These genes were mostly related to signal transduction and  
141 the response to stimulus, which could be related to the previously described different susceptibility  
142 to CD3 activation of the CD4+ T cell subsets (Croft et al., 1994; Kumar et al., 2011). We found that the  
143 level of HIV-infected cells correlated with the expression of several genes at the time of infection in  
144 the different conditions studied (Figure 2B and Figure S2). SAMHD1 showed a negative association  
145 with infection. In contrast, positive correlations were observed between infection levels and other  
146 antiviral factors (such as APOBEC3G or SLFN11)(Li et al., 2012; Sheehy et al., 2002) as well as several  
147 genes involved in the interferon response (IFI6, IFI16, EIF2AK2, and OAS1) (Kane et al., 2016).  
148 Significant positive correlations were also observed between the level of HIV infection and the gene  
149 expression levels of transcription factors (STAT3, E2F1, and PRDM1), genes that have been proposed  
150 to facilitate HIV-1 infection (RRM2, HSP90AA, CFL1, and DYNC1H1)(Allouch et al., 2013; Franke and  
151 Luban, 1996; Lukic et al., 2014; Roesch et al., 2012) and multiple genes involved in T cell metabolism  
152 (SLC2A3, SLC2A1, SLC2A5, CASP3, FAS, GAPDH, and GUSB). Taken together, these results suggest

153 that, with the exception of SAMHD1, the antiviral restriction factors analyzed did not decisively  
154 influence the cell susceptibility to HIV-1, which is in line with the results of previous reports (Jia et al.,  
155 2015). Interestingly, our data indicate that metabolically active cells may offer favorable conditions  
156 for HIV infection.

157

### 158 **Hierarchy of susceptibility to HIV infection matches metabolic activity of CD4+ T cell subsets**

159 To explore the possible association between HIV infection and cell metabolism, we first determined  
160 the metabolic activity of the CD4+ T cell subsets at the time of infection. We used a cell flux analyzer  
161 to measure, in different conditions, the oxygen consumption rate (OCR) and the extracellular  
162 acidification rate (ECAR) as indicators of oxidative phosphorylation (OXPHOS) and glycolysis,  
163 respectively (Zhang et al., 2012). In the absence of activation in vitro and in agreement with their  
164 quiescent nature, all sorted CD4+ T cell subsets had low levels of metabolic activity (Figure 3A).  
165 Nonetheless, small differences between subsets were noted; basal metabolism and metabolic  
166 potential were highest in Tem cells and lowest in Tn cells, while Ttm and Tcm cells presented similar  
167 intermediate levels (Figures 3A, B). These differences were more pronounced after activation, with  
168 all memory cell subsets increasing mitochondrial function and glycolysis to different extents and with  
169 different kinetics. The highest metabolic activity was measured in Tem cells, peaking on day 3 after  
170 activation and decreasing on day 5. The metabolism of Ttm and Tcm cells increased after 3 days of  
171 activation and then remained stable in Ttm cells while continuing to increase in Tcm cells. In contrast,  
172 Tn cells showed a modest increase only in mitochondrial function and not in glycolysis and only after  
173 5 days of activation, when their metabolism was heavily relying on OXPHOS (Figure 3C). Accordingly,  
174 important differences were also found between CD4+ T cell subsets regarding their capacity to  
175 uptake glucose and their levels of the surface expression of the Glut1 receptor as analyzed by flow  
176 cytometry, which were lowest in Tn cells and highest in Tem cells (Figures S3 and S4). The relative  
177 metabolic activity levels of the different cell subsets matched their relative susceptibility to HIV-1  
178 infection (Figure 1C), and we found positive correlations between HIV infection levels and multiple

179 metabolic functions in cells that had been activated (Figure 3D, Figure S5). These results further point  
180 to an influence of the metabolic activity of CD4+ T cells on their susceptibility to HIV-1.

181

182 **HIV-infected CD4+ T cells are characterized by higher levels of metabolic activity independent of**  
183 **cell activation levels**

184 To analyze if there was a direct link between cell metabolism and HIV-1 infection, we challenged 5-  
185 day activated bulk CD4+ T cells with VSV-G pseudotyped NL4.3ΔenvGFP particles, and we sorted  
186 noninfected GFP- and infected GFP+ cells three days later to analyze their metabolic activity levels.  
187 HIV-infected CD4+ T cells had much higher levels of basal metabolism and metabolic potential and,  
188 overall, a more energetic profile than noninfected cells (Figures 4A and S6). Although we could detect  
189 a significant fraction of infected cells among cells with low activation levels (Figure 4B), we found  
190 higher proportions of GFP+ cells among CD4+ T cells expressing activation markers. We therefore  
191 evaluated whether differences in the metabolic activity of infected and noninfected CD4+ T cells  
192 were just a consequence of the selective infection of CD4+ T cells with higher activation levels. We  
193 sorted CD4+ T cells first based on their expression of either high or low levels of both HLA-DR and  
194 CD25 and then based on whether they were GFP+ or GFP- (Figures 4B and S7). After 5 days of  
195 stimulation, the CD4+ T cell subsets expressed different levels of activation markers (Figure S7A),  
196 which were highest in Tem cells and lowest in Tn cells. This was translated to different contributions  
197 of CD4+ T cell subpopulations in the high- and low-activation sorted cell fractions (Figure 4B).  
198 Nevertheless, Tn cells were more frequently found in the GFP- fraction, both in high and low-  
199 activated cell populations, whereas the GFP+ fraction was enriched with Tem cells. These results  
200 matched the hierarchy of infection that we observed before (Figure 1) and further supported that  
201 the susceptibility of CD4+ T cell subsets to HIV-1 depends on the intrinsic characteristics of these cells  
202 independent of their activation status. In this regard, we found that infected GFP+ cells in both the  
203 high- and low-activation fractions, had higher basal metabolisms (OCR and ECAR) than noninfected

204 GFP- cells (Figure 4C). These results demonstrated that HIV-infected CD4+ T-cells were characterized  
205 by higher metabolic activity levels.

206

### 207 **HIV-1 infection is preferentially established in CD4+ T cells with high metabolic activity levels**

208 Our results suggest that HIV-1 infection is favored in the environment provided by CD4+ T cells with  
209 high metabolic activity levels. We analyzed if this was due to a selective infection of CD4+ T cells with  
210 the highest metabolic activity levels or if it was HIV-infection that increased the metabolic activity of  
211 the cells. We activated CD4+ T cells and sorted Tn and Tcm cells based on their capacity to uptake  
212 high or low levels of the fluorescent glucose analogue 2NBDG (Figure S8A), which corresponded to  
213 weakly and strongly glycolytic cells (Figure S8B). We infected these purified cell fractions with VSV-G  
214 pseudotyped NL4.3ΔenvGFP particles. Three days after infection, infected GFP+ CD4+ T cells were  
215 only observed among highly glycolytic Tn and Tcm cells, while weakly glycolytic cells were strongly  
216 resistant to infection (65x [41x-206x], median [IQR] fold increase in the proportion of GFP+ cells in  
217 HGlu vs LGlu cell subsets,  $p=0.008$ ) (Figure 5). Overall these results confirmed that, in our conditions,  
218 the high metabolic activity of infected CD4+ T cells was one of the causes rather than a consequence  
219 of HIV infection.

220

### 221 **Suboptimal inhibition of glucose metabolism blocks HIV-1 replication in CD4+ T cells**

222 The above results indicate that HIV-1 infection of CD4+ T cells required high levels of metabolic  
223 activity. Therefore, we analyzed if HIV-1 replication could be blocked with metabolic inhibitors. We  
224 infected activated CD4+ T cells with VSV-G pseudotyped NL4.3ΔenvGFP particles in the presence of  
225 increasing amounts of etomoxir, an inhibitor of fatty acid oxidation (FAO), 6-diazo-5-oxo-l-norleucine  
226 (DON), a glutamine antagonist, or 2-deoxy glucose (2-DG), a competitive inhibitor of glycolysis (Figure  
227 S9). Etomoxir was able to reduce HIV infection but only at high concentrations, well above the levels  
228 needed to reduce mitochondrial respiration without inducing cell death and at concentrations known  
229 to induce off target effects (O'Connor et al., 2018; Yao et al., 2018)(Figure 6A and Figure S9). DON

230 reduced HIV infection without inducing cell death, although the extent of the inhibition was  
231 heterogeneous. Suboptimal amounts of 2-DG (e.g., 5 mM), which were enough to significantly  
232 reduce glycolysis (Figure S9), decreased HIV-1 infection of CD4+ T cells with minimal cell toxicity  
233 (Figure 6A). These results suggested a higher impact of glucose and glutamine metabolism than FAO  
234 on HIV-1 replication. The role of glucose metabolism was further confirmed in different sets of  
235 experiments in which the frequency of HIV-1-infected CD4+ T cells was reduced when the infections  
236 were performed in conditions of glucose starvation or in presence of UK5099, a molecule that  
237 inhibits the transport of pyruvate, an end product of glycolysis, to the mitochondria (Figure 6B). The  
238 presence of 2-DG impaired the accumulation of HIV-1 reverse transcribed products overtime pointing  
239 to an early block of viral replication (Figure 6C). Of note, 2-DG was able to reduce infection and  
240 reverse transcript levels to a similar extent whether it was added to the culture at the time of the  
241 challenge or up to 8h later (Figure 6D), indicating that 2-DG was affecting post-entry steps of viral  
242 replication. Overall, these results show that a glycolytic environment was necessary for HIV-1 to  
243 complete reverse transcription.

244  
245 2-DG blocked HIV-1 infection in all CD4+ T cell subsets, although the differences were more  
246 pronounced in more differentiated (more glycolytic) cells (Figure 6E). Interestingly, Etomoxir slightly  
247 reduced viral replication in Tem cells but not in other T cell subsets, which could be related to the  
248 overall highly energetic nature of these cells. We then used VSV-G pseudotyped NL4.3Δenv Duo-Fluo  
249 I particles that allow HIV-1 latently and productively infected cells to be distinguished from each  
250 other (Calvanese et al., 2013; Chavez et al., 2015) (Figure S10A). Interestingly, latent infection was  
251 more prominent among Tn and Tcm CD4+ T cells, while productive infection was predominantly  
252 observed among Tem cells (Figure S10B). Overall, the presence of 2-DG significantly reduced the  
253 global number of both latently and productively infected CD4+ T cells (Figure 6F), which agreed with  
254 the need for a glycolytic environment for HIV-1 to complete the preintegration steps of its replication  
255 cycle.

256 Although our results indicated that the effect of 2-DG was not related to the entry of the VSV-G  
257 pseudotyped viral particles through the endocytic pathway, we nevertheless analyzed the impact of  
258 inhibition of glycolysis on the infection of CD4+ T cells with a R5 wild-type replication competent  
259 virus (HIV-1 Bal). We first confirmed that the hierarchy of infection of CD4+ T cell subsets that we  
260 observed with VSV-G single cycle particles (Tn<Tcm<Ttm<Tem) coincided with the hierarchy of  
261 infection when we used replication competent HIV-1 Bal (Figure S11A). Next, we found that 2-DG  
262 was also able to efficiently blocked infection of CD4+ T cells with HIV-1 Bal (Figure 6G), independently  
263 of whether it was added at the time of challenge or 4h/8h after challenge (Figure S11B). All together,  
264 these results show that the inhibition of metabolic activity blocked HIV-1 replication, corroborating  
265 that CD4+ T cell metabolism is an important determinant of HIV-1 infection.

266

#### 267 **Suboptimal inhibition of glycolysis eliminates HIV-1 infected cells and impairs HIV amplification** 268 **from CD4+ T cell reservoirs**

269 We next studied if the preferential establishment of HIV-1 infection in highly glycolytic cells could be  
270 used to target HIV-1 reservoirs. First, we analyzed if suboptimal inhibition of glycolysis could  
271 selectively eliminate CD4+ T cells that had been preinfected in vitro. We infected CD4+ T cells with  
272 VSV-G pseudotyped NL4.3ΔenvGFP particles for three days and sorted infected GFP+ from  
273 noninfected GFP- cells (Figure S6) and cultured them in the absence or presence of 2-DG to inhibit  
274 glycolysis. The presence of 2-DG induced much higher levels of cell death among infected GFP+ cells  
275 than among GFP- cells (Figure 7A and B), affecting all memory T cell subpopulations (Figure 7C).

276

277 As 2-DG was able to both block infection and eliminate infected cells, we wondered whether 2-DG  
278 could block HIV spread upon activation of CD4+ T cells from HIV-infected individuals receiving cART.  
279 We isolated CD4+ T cells from 6 individuals receiving cART (Table S2) and activated the cells with PHA  
280 in the absence or presence of 2-DG. In all cases, 2-DG potently blocked HIV-1 amplification, as  
281 measured by ultrasensitive analyses of p24 production in the culture supernatants (Figure 7D).



282 Therefore, the need of HIV for highly glycolytic cells reveals a vulnerability that can be exploited to  
283 tackle infection.  
284

285 **DISCUSSION**

286 We show here that susceptibility to HIV-1 infection is affected by the different metabolic  
287 programming of CD4+ T cell subsets. In particular, we have found that HIV-1 selectively completes its  
288 replication cycle in those CD4+ T cells with the highest levels of metabolism. Consequently,  
289 suboptimal inhibition of glycolysis both impaired infection in vitro and selectively eliminated infected  
290 cells. Moreover, mild inhibition of glycolysis potently blocked HIV reactivation in CD4+ T cell  
291 reservoirs from HIV-infected individuals on antiretroviral therapy.

292

293 In this study, we performed a detailed characterization of the bioenergetics of CD4+ T<sub>n</sub>, T<sub>cm</sub>, T<sub>tm</sub>  
294 and T<sub>em</sub> cells. Upon potent TCR activation, naïve and memory cells have been shown to strongly  
295 upregulate their metabolism and acquire effector functions (van der Windt et al., 2013). Here, we  
296 show important metabolic differences among the three memory cell populations studied, even in the  
297 absence of stimulation. Upon anti-CD3 activation, all CD4+ T cell subsets enhanced their metabolic  
298 activity but essentially maintained their distinctive metabolic programs, which matched the  
299 requirements for their expected rapid reaction to antigenic stimulation (T<sub>em</sub>>>T<sub>tm</sub>>T<sub>cm</sub>>>T<sub>n</sub>). The  
300 metabolic activity of the T cell subsets overlapped with their susceptibility to HIV-1 infection (Figures  
301 1C and 3B), supporting that the extent of HIV-1 infection in CD4+ T cell subsets was affected by the  
302 metabolic environment within the target cells.

303

304 Transcript profiling at the time of infection showed that among the CD4+ T cell subsets, there were  
305 positive correlations between the frequencies of HIV-infected cells and the expression levels of  
306 multiple genes related to cell metabolism. Negative correlations were found between the  
307 susceptibility of CD4+ T cells to HIV-1 infection and the expression of SAMHD1, an efficient HIV-1  
308 restriction factor that also plays an important role in the regulation of cell metabolism (Descours et  
309 al., 2012; Laguette et al., 2011; Mathews, 2015). Surprisingly, strong positive correlations were found  
310 between the levels of HIV-infected cells and the expression of a cluster of genes related to the

311 interferon response. Although this point was not specifically explored in the present study, increasing  
312 evidence has revealed the interrelationships between cell metabolism and the interferon response  
313 (Ahmed and Cassol, 2017; Burke et al., 2014; Zhao et al., 2015). Some type 1 interferons might  
314 enhance glycolysis (Fritsch and Weichhart, 2016), and interferon regulatory factors play a key role  
315 during the metabolic reprogramming that follows TCR-mediated activation of T cells (Man et al.,  
316 2013). The interaction between the interferon response and cell metabolism may somewhat explain  
317 the dichotomy between antiviral and viral-enhancing interferon-stimulated genes (Schoggins and  
318 Rice, 2011; Seo et al., 2011). Tem cells, which were the most susceptible to HIV infection in our assay,  
319 expressed the strongest levels of several restriction factors such as SLFN11 or APOBEC3G. Our results  
320 thus indicate that HIV-1 exploits the metabolic environment that most favors the completion of its  
321 replication cycle, and this might be one of the factors underlying the adaptation of HIV-1 to evade  
322 some restriction factors.

323

324 We further confirmed the association between T cell metabolism and HIV infection in a series of  
325 functional analyses. First, we showed that HIV-infected CD4+ T cells had higher levels of metabolic  
326 activity and metabolic potential than HIV-exposed but noninfected cells. This was not solely the  
327 consequence of the preferential infection of cells with higher activation levels; when we sorted CD4+  
328 T cells that were matched for the expression of common activation markers, we still found that HIV-  
329 infected cells had higher metabolic activity levels than noninfected CD4+ T cells. Although there are  
330 well-established links between T cell activation and cellular metabolism, it is increasingly clear that T  
331 cell functions, including proliferation, the secretion of cytokines and cell survival, are supported  
332 through different engagements of the various metabolic pathways (Jones and Bianchi, 2015). This  
333 may explain the partial dichotomy between T cell activation and cell metabolism in HIV infection that  
334 we observed in our experiments. Additionally, we found Tn cells expressing high levels of activation  
335 markers upon anti-CD3 stimulation, but these cells remained mostly resistant to HIV-1 infection. In  
336 contrast, the frequency of infected Tn cells sharply increased when we challenged highly glycolytic Tn

337 cells. This is in agreement with previous results that showed that expression of Glut-1 is necessary for  
338 HIV-1 infection of CD4+ T cells (Loisel-Meyer et al., 2012) and that, in vitro, HIV preferentially infects  
339 CD4+ T cells expressing Glut-1 and OX40 (Palmer et al.). Overall, our results demonstrate that cells  
340 that had higher metabolic activity levels were more susceptible to HIV infection.

341

342 In our experimental conditions, we could detect virtually no infected cells when we challenged cells  
343 with low metabolic activity levels. Thus, any potential change in cell metabolism that might have  
344 been induced directly by HIV particles was not sufficient to promote infection in cells that had low  
345 metabolic activity levels at the time of viral challenge. However, it is important to note that because  
346 we were interested in understanding the factors modulating HIV infection beyond the expression of  
347 HIV receptors, we used single-cycle particles devoid of HIV envelope and pseudotyped with VSV-G in  
348 this set of experiments. It is possible that fully replication-competent viruses have a stronger effect  
349 on modulating CD4+ T cell metabolism. CCL5 engagement with CCR5 has been described as  
350 increasing glycolysis in T cells (Chan et al., 2012), and it is possible that gp120 triggers a similar effect.  
351 Moreover, HIV infection has been shown to induce increased expression of several glucose  
352 transporters in in vitro experiments (Kavanagh Williamson et al., 2018; Sorbara et al., 1996). HIV-  
353 infected individuals carry higher frequencies of Glut1+ CD4+ T cells, and these levels are not  
354 normalized after the initiation of antiretroviral therapy (Palmer et al., 2014). Overall, viruses appear  
355 to possess different mechanisms to enhance cell metabolism to favor viral replication (Goodwin et  
356 al., 2015; Sanchez and Lagunoff, 2015), and this deserves additional exploration in the context of HIV  
357 infection.

358

359 Suboptimal inhibition of glycolysis impaired HIV replication, and this was observed with single-cycle  
360 VSVG pseudotyped particles and replication-competent HIV-1 Bal and for all CD4+ T cell subsets,  
361 although the effects were more pronounced in more energetic cells. Inhibition of glycolysis, including  
362 several hours after viral entry, severely reduced the accumulation of HIV reverse transcripts and

363 impaired the establishment of both productive and latent infections. Our results thus point to critical  
364 steps early during the viral replication cycle (in particular reverse transcription) that are influenced by  
365 glycolysis, which agrees with a previous report (Loisel-Meyer et al., 2012). Along these lines, the  
366 synthesis of deoxynucleotides, the level of which is a limiting factor for HIV reverse transcription, is  
367 very energy demanding and requires substrates that are provided by different metabolic pathways,  
368 such as the pentose phosphate pathway that is parallel to glycolysis (Lane and Fan, 2015; Mathews,  
369 2015). Although, unfortunately, genes involved in the PPP were not included in our gene expression  
370 panel, we found important differences between CD4+ T cell subsets and strong correlations with  
371 infection levels for several genes such as TP53, ESF1 and RRM2, which play critical roles in the de  
372 novo synthesis of dNTPs. In particular we have recently shown that changes in the expression of  
373 RRM2 impact HIV-1 replication in macrophages and dendritic cells by modifying the pools of dNTPS  
374 (Allouch et al., 2013; Valle-Casuso et al., 2017). Moreover, SAMHD1, the expression levels of which  
375 were negatively correlated with infection in our analysis, is a deoxynucleoside triphosphohydrolase  
376 that contributes to control the intracellular dNTP concentration during cell-cycle (Mathews, 2015).  
377 Our results therefore suggest that metabolically active cells offer an environment with positive  
378 synthesis (RRM2) vs degradation (SAMHD1) of dNTP pools that favors HIV-1 reverse transcription.  
379 However, other steps of the viral replication cycle may also depend on cell metabolism. The  
380 inhibition of glycolysis has been shown to decrease the production of HIV-1 particles (Hegedus et al.,  
381 2014), and mTOR, a key regulator of cellular metabolism (Waickman and Powell, 2012), appears to  
382 be involved in the establishment of HIV-1 latency in CD4+ T cells (Besnard et al., 2016).

383

384 In our functional experiments we mostly focused on assessing the impact of glycolysis on HIV  
385 infection. Our results showing that inhibition of pyruvate transport to the mitochondria with UK5099  
386 blocked HIV infection suggests that glucose oxidation is important for HIV-1 infection. However the  
387 relative contribution of aerobic vs oxidative glycolysis remains to be determined. It is likely that other  
388 metabolic functions are also important for HIV-1 infection. The inhibition of fatty acid oxidation with

389 Etomoxir had a limited effect on HIV replication in suboptimal conditions, mostly in Tem cells, but it  
390 strongly inhibited infection at higher concentrations. However, caution is needed when interpreting  
391 results obtained with Etomoxir as it has been shown to produce off target effects at such high  
392 concentrations (O'Connor et al., 2018; Yao et al., 2018). A recent report suggested that fatty acid  
393 metabolism may also participate in the late steps of viral replication (Kulkarni et al., 2017). Our  
394 results with the glutamine antagonist DON suggest that glutamine metabolism may also be necessary  
395 for the optimal infection of CD4+ T cells. In general, the association between HIV infection and cell  
396 metabolism can be exploited to impair HIV-1 replication.

397

398 Cell survival is another process regulated by cell metabolism that could be critically relevant for the  
399 persistence of infected cells. We found that suboptimal inhibition of glycolysis induced the selective  
400 death of cells that had been preinfected in vitro, and this affected all CD4+ T cell memory subsets. It  
401 remains to be determined if HIV-infected cells sustain enhanced metabolic activity levels over time,  
402 even in the absence of active viral replication and if this could serve to identify cells carrying  
403 proviruses. CD4+ T cells expressing PD-1 and other immune checkpoints are enriched in HIV in HIV-  
404 infected individuals receiving cART (Banga et al., 2016; Chomont et al., 2009; Fromentin et al., 2016).  
405 Interestingly, these immune checkpoints appear to mediate their inhibitory activities through the  
406 metabolic reprogramming of the cells (Herbel et al., 2016; Lim et al., 2017; Patsoukis et al., 2015). We  
407 show here that the partial inhibition of glycolysis in CD4+ T cells from HIV-infected individuals on  
408 cART potently blocked viral reactivation and spread. Based on our results, this could be the result of a  
409 combination of both the elimination of infected cells and the blocking of new cycles of viral  
410 amplification by 2-DG. Overall our results point to the potential modulation of cell metabolism as a  
411 strategy to combat HIV infection.

412

413 Therapies targeting cellular metabolism are gaining interest in the cancer field (Hanahan and  
414 Weinberg, 2011; Zhao et al., 2013). Of note, metabolic reprogramming observed in tumor cells

415 closely resembles the metabolic profile of HIV-infected T cells that we describe here. In the context  
416 of the physiopathology of HIV infection, high glucose consumption by infected CD4+ T cells could  
417 have additional implications for immune responses. We recently found that while HIV-specific CD8+ T  
418 cells from rare individuals naturally controlling HIV infection are characterized by metabolic plasticity,  
419 HIV-specific CD8+ T cells from most HIV-infected subjects heavily rely on glycolysis to exert their  
420 functions (Angin *et al*, submitted). High levels of glucose consumption by CD4+ T cells at the sites of  
421 viral replication might severely limit glucose availability for these CD8+ T cells and impair their  
422 effector function. In addition, lactic acid, which is a product of glycolysis, inhibits effector functions in  
423 cytotoxic T cells (Mendler et al., 2012). Therefore, the metabolic characteristics of HIV-infected CD4+  
424 T cells may provide the virus with additional mechanisms to mediate immune evasion, as has also  
425 been described for tumors (Sugiura and Rathmell, 2018). Because exploiting the host cell metabolic  
426 machinery appears to be a common strategy for invading pathogens, including viruses, bacteria and  
427 parasites, therapies targeting cell metabolism could affect a large spectrum of infections. Obviously,  
428 cell metabolism regulates critical physiological events, including immune responses, and it is  
429 necessary to develop a better understanding the links between cell metabolism and acute and  
430 chronic infections. Overall, our study shows that cellular metabolism is a central factor that drives  
431 the HIV-1 infection of CD4+ T cells more strongly than does the state of differentiation and/or  
432 activation, and cellular metabolism may be an important target for new therapies against HIV-1.  
433

434

## 435 **MATERIALS AND METHODS**

### 436 **Subjects**

437 Blood samples from non-HIV-infected donors were obtained from the French blood bank  
438 (Etablissement Français du Sang) as part of an agreement with the Institut Pasteur (C CPSL UNT,  
439 number 15/EFS/023). Fifty-milliliter blood samples were obtained from six HIV-infected individuals  
440 on antiretroviral therapy who had HIV plasma viral loads <50 RNA copies/mL from the ANRS  
441 TRANSbioHIV study after obtaining written informed consent in accordance with the Declaration of  
442 Helsinki (Table S2). The TRANSbioHIV study was approved by the Ethics Review Committee (Comité  
443 de protection des personnes) of Île-de-France VII.

444

### 445 **Isolation and culture of CD4+ T cells**

446 CD4+ T cells were purified (>90%) from freshly isolated PBMCs by negative selection with antibody-  
447 coated magnetic beads (EasySep™ Human CD4+ T Cell Enrichment Kit Ref.19052) in a Robosep  
448 instrument (Stem Cell Technology).

449 Purified CD4+ cells ( $10^6$  cell/mL) were cultured in RPMI 1640 containing GlutaMAX, 10% FCS,  
450 penicillin (10 IU/mL) and streptomycin (10  $\mu$ g/mL) in the presence of IL-2 (Miltenyi) at 50 IU/mL  
451 (Culture media). Depending on the experiment, cells were left unstimulated or were stimulated for 3  
452 or 5 days with 0.5  $\mu$ g/mL soluble antiCD3 (BioLegend, Ref.300414, Clone UCHT1) in the absence of  
453 CD28 co-stimulation as previously described (Saez-Cirion et al., 2011). Different compounds that  
454 target metabolic pathways [2-deoxy-glucose, 2-DG (Seahorse Biotechnologies); (+)-Etomoxir sodium  
455 salt hydrate (Sigma, Ref. E1905); UK5099 (Sigma, Ref. PZ0160); 6-diazo-5-oxo-l-norleucine  
456 (DON)(Sigma, Ref. D2141); glucose (Seahorse Biotechnologies); oligomycin (Seahorse  
457 Biotechnologies) or carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone FCCP (Seahorse  
458 Biotechnologies)] were added to the culture media at different times and concentrations depending  
459 on the protocol conditions. A glucose-free culture media was used in some infection experiments and



460 is described in the results section [RPMI non-glucose, GlutaMAX, containing 10% FCS, penicillin (10  
461 IU/mL) and streptomycin (10 µg/mL) in the presence of IL-2 (Miltényi) at 50 IU/mL (culture media)].  
462 After culture, living cells were counted with an automatic Countess cell counter (Invitrogen) based on  
463 size and non-staining with trypan blue. The number of living cells was then normalized before  
464 analysis.

465

#### 466 **HIV infection in vitro**

467 Single-round infections were performed with HIV-1 NL4.3ΔenvΔnef/GFP (Amara et al., 2003) and  
468 HIV-1-DuoFluoΔenv(R7GEmC) (provided by Professor Eric Verdin and Dr. Calvanese, NIH AIDS  
469 Reagent Program, Division of AIDS, NIAID, NIH: Cat# 12595 DuoFluo (R7GEmC)) (Calvanese et al.,  
470 2013). Both viruses were pseudotyped with the VSV-G envelope protein as previously described  
471 (David et al., 2006). Nonactivated or activated CD4 + T cells were infected in triplicate ( $5 \times 10^4$   
472 cells/well, 200 µL) with 35 ng/ $1 \times 10^6$  HIV-1 NL4.3Δnef/GFP/VSV-G and with 70 ng of HIV-1-  
473 DuoFluo(R7GEmC)/VSVg per million cells. Active HIV-1 infection was estimated by flow cytometry  
474 (BD LSRII, BD bioscience) as the percentage of GFP-expressing CD4+ T cells 72 h after infection. Latent  
475 HIV infection was estimated by flow cytometry as the percentage of mCherry+GFP-CD4+ T cells 72 h  
476 after infection with HIV-1-DuoFluo(R7GEmC) particles.

477

478 HIV-1 reverse transcripts (U5-Gag) were quantified by real-time PCR with an Applied Biosystems 7500  
479 Real-Time PCR System (as described in (David et al., 2006)) 6, 16 and 72 h after infection of CD4+ T  
480 cells with VSV-G–pseudotyped HIV-1 particles. Total DNA was extracted with the NucleoSpin 8/96  
481 Tissue Core kit (Macherey-Nagel, Ref. 740453.4). DNA loading was controlled by concurrently  
482 amplifying the albumin gene by real-time PCR and quantifying with reference to a control human  
483 genomic DNA (Roche).

484

485 Productive HIV-1 infection in vitro was studied in suboptimally activated CD4<sup>+</sup> T cells (10<sup>6</sup> cells/mL in  
486 triplicate) exposed to the HIV-1 BaL strain (R5) (10 ng p24/ml). The cells were cultured in 96-U-well  
487 plates for 14 days in the presence or absence of 2-DG (5 mM). Every 3-4 days, the culture  
488 supernatants were removed and replaced with fresh culture medium with or without 2-DG. Viral  
489 replication was monitored in the supernatants by p24 enzyme-linked immunosorbent assay (ELISA)  
490 (XpressBio) or at day 3 by intracellular p24 staining (p24-FITC (clone KC57, Coulter) (Saez-Cirion et al.,  
491 2010).

492

#### 493 **Flow-assisted sorting of CD4 + T cell subsets**

494 Cells were first selected based on size and structure to eliminate cellular debris. Then cell singlets  
495 and living cells (not stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, Thermofisher) are gated  
496 before proceeding with further selection based on phenotypical or functional markers (Figure S8).

497

498 Resting (CD25<sup>-</sup>, CD69<sup>-</sup>, HLA-DR<sup>-</sup>) CD4<sup>+</sup> T cell subsets [naïve (**T<sub>n</sub>**; CD3<sup>+</sup>, CD4<sup>+</sup>, CD45RA<sup>+</sup>, CCR7<sup>+</sup>,  
499 CD27<sup>+</sup>, CD95<sup>-</sup>), central memory (**T<sub>cm</sub>**; CD3<sup>+</sup>, CD4<sup>+</sup>, CD45RA<sup>-</sup>, CCR7<sup>+</sup>, CD27<sup>+</sup>), transitional memory  
500 (**T<sub>tm</sub>**; CD3<sup>+</sup>, CD4<sup>+</sup>, CD45RA<sup>-</sup>, CCR7<sup>-</sup>, CD27<sup>+</sup>) or effector memory (**T<sub>em</sub>**; CD3<sup>+</sup>, CD4<sup>+</sup>, CD45RA<sup>-</sup>, CCR7<sup>-</sup>,  
501 CD27<sup>-</sup>)] were sorted on a FACS ARIA III cell sorter (BD) using the following antibody panel: CD3-  
502 eFluor450 (eBioscience), CD4-alexaFluor700 (BD), CD45RA-ECD (BC), CCR7-PE\_Cy7 (BioLegend),  
503 CD27-APC (Miltenyi), CD95-PE (Miltenyi), CD25-FITC (BD), CD69-FITC (eBioscience) and HLA-DR-FITC  
504 (BD). The gating strategy is depicted in Figure S1. The number of sorted cells varied from 0.5 to 5  
505 million cells depending on the CD4<sup>+</sup> T cell subset and the donor. The purity of the sorted subset was  
506 greater than 98%.

507

508 GFP<sup>+</sup> and GFP<sup>-</sup> CD4<sup>+</sup> T cells were sorted 72 h after infection with VSV-G pseudotyped  
509 NL4.3ΔenvΔnef/GFP particles (Figure S6). For some experiments, GFP<sup>+</sup> and GFP<sup>-</sup> cells were also  
510 sorted into the following categories based on their expression of activation markers (CD25-ECD, HLA-

511 DR\_PerCyP5.5) (Figure 4 and Figure S7): high activation GFP+ [**H/+** (GFP+, CD25+,HLA-DR+)]; high  
512 activation GFP- [**H/-** (GFP-, CD25+, HLA-DR+)]; low activation GFP+ [**L/+**, (GFP+, CD25-,HLA-DR-)]; and  
513 low activation GFP- [**L/-** (GFP-, CD25-,HLA-DR-)].

514

515 For some experiments, CD4+ Tn and Tcm cells were sorted based on their level of glucose uptake  
516 after 5 days of stimulation with anti-CD3 (Figure S8). The cells were washed and incubated with 2-  
517 NBDG (2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose) (Thermo Fisher, Ref. N13195)  
518 at 75  $\mu$ M in PBS for 30 min at 37°C. After 3 washes of 10 min each with fresh PBS, the cells were  
519 stained with antibodies (CD3-eFLuor450 (eBioscience), CD4-alexaFluor700 (BD), CD45RA-ECD (BC),  
520 CCR7-PE\_Cy7 (BioLegend), and CD27-APC (Miltenyi)) and sorted as follows: **Tn HGlu** (CD3+, CD4+,  
521 CD45RA+, CCR7+, CD27+, 2NBDG+); **Tn LGlu** (CD3+, CD4+, CD45RA+, CCR7+, CD27+, 2NBDG-); **Tcm**  
522 **HGlu** (CD3+, CD4+, CD45RA-, CCR7+, CD27+, 2NBDG+); and **Tcm LGlu** (CD3+, CD4+, CD45RA-, CCR7+,  
523 CD27+, 2NBDG-).

524

#### 525 **Quantitative RT-PCR arrays**

526 The expression levels of an array of 96 genes in the CD4+ T cell subsets were quantified by RT-qPCR  
527 with a Biomark HQ system. Total RNA was extracted from  $5 \times 10^4$  CD4+ T cells with an RNA trace kit  
528 (Macherey-Nagel, Ref. 740731.4) and treated with DNase, following the manufacturer's instructions.  
529 Twenty microliters of RNA (> 10 ng) was reverse transcribed with Reverse Transcription Master Mix  
530 (Fluidigm, 100-6298) (5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C). A specific target  
531 preamplification (STA) was performed by adding PreAmp Master Mix, 96 Primers Mix and EDTA to  
532 the cDNA, followed by STA cycling (95°C: 2 min, 18 cycles of [96°C: 5 s, 60°C 4 min]). The sample was  
533 then treated with exonuclease I (New England Biolabs) (37°C: 30 min, 80°C: 15 min). Sample premix  
534 (SsoFast EvaGreen Supermix with Low ROX (Biorad), DNA Binding Dye (Fluidigm), preamplified Exo 1-  
535 treated sample) and assay mix (assay loading reagent (Fluidigm), Delta Gene primers (Fluidigm)) were  
536 then loaded on primed 96.96 Dynamic Array chips (Fluidigm). The chips were transferred into a

537 Biomark HQ device (Fluidigm) for thermocycling, and fluorescence was acquired with the GE 96×96  
538 PCR+Melt v2 program. Linear derivative mode baseline correction was applied. We used the  
539 Normfinder algorithm (Aarhus University Hospital, Denmark) (Andersen et al., 2004) to identify the  
540 optimal normalization gene among the assayed candidates for our experimental conditions. BENC1  
541 was thus identified as the optimal normalization gene based on expression stability in the analyzed  
542 samples (Table S3), and the gene expression values were plotted as  $2^{-\Delta\Delta Ct}$ , where  $\Delta\Delta Ct = \Delta Ct_{\text{SAMPLE}} -$   
543  $\Delta Ct_{\text{CONTROL}}$ , and  $\Delta Ct = Ct_{\text{TARGET GENE}} - Ct_{\text{BENC1}}$ .

544

#### 545 **Measurement of oxygen consumption and extracellular acidification rates**

546 The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using  
547 a Seahorse XF96 metabolic analyzer following the procedure recommended by the manufacturer.  
548 Briefly, for all the experiments, different CD4<sup>+</sup> T cell populations were seeded at a concentration of  
549  $2 \times 10^5$  cells per well on XF96 plates (Seahorse Bioscience) precoated with 0.5 mg/ml Cell Tack  
550 (Corning, Ref. 354240) immediately before adding Seahorse XF culture media to each well. Cells were  
551 incubated for 50 min in a CO<sub>2</sub>-free incubator at 37°C before loading the plate in the Seahorse  
552 analyzer. Different programs were run on the Seahorse analyzer depending on the assay. Drug Panel  
553 A (1) XFmedia 2) oligomycin (2.5 μM), 3) FCCP (0.9 μM) and 4) rotenone (1 μM) and antimycin A (1  
554 μM)) was injected through ports A, B, C and D, respectively, for the mitochondrial stress test. Drug  
555 Panel B ( 1) XFmedia 2) glucose (10 mM) 3) oligomycin (2.65 μM), and 4) 2-DG (100 mM)) was used  
556 for the glycolysis stress test.

557

#### 558 **Phenotyping after sorting**

559 In some experiments, sorted GFP<sup>±</sup> CD4<sup>+</sup> T cells subset or CD4<sup>+</sup> T bulk cells previously infected with  
560 NL4.3Δnef/GFP/VSV-G with or without 2-DG or Etomoxir were incubated with CD3-eFLuor450  
561 (eBioscience), CD4-alexaFluor700 (BD Biosciences), CD45RA-ECD (BC), CCR7-PE\_Cy7 (BioLegend) and  
562 CD27-APC (Miltenyi) to determine the CD4<sup>+</sup> T cell subset distribution. In addition, the activation

563 levels of sorted CD4+ T cell subsets were assessed with CD25-ECD (BD Biosciences) and HLA-DR-FITC  
564 (BD Biosciences). For both protocols, cells were incubated with the antibodies for 25 minutes and  
565 then washed in PBS plus 1% FCS and fixed in 4% paraformaldehyde for flow cytometry on an LSRII  
566 device (BD Biosciences). The data were analyzed with Kaluza software (Beckman Coulter).

567

#### 568 **HIV-1 reactivation in CD4+ T cells from HIV-1-infected individuals.**

569 Freshly isolated CD4+T cells (negative selection kit, Stem Cell) from HIV-1-infected individuals undergoing  
570 successful cART were seeded in 48-well plates ( $1 \times 10^6$  cells/well, in triplicate) and stimulated with  
571 phytohemagglutinin-L (PHA-L, Roche, 1  $\mu\text{g}/\text{mL}$ ) and IL-2 (Miltenyi) 100UI with or without 2-DG (5  
572 mM). The culture supernatants were collected every 3 to 4 days, and fresh medium +/- 2-DG was  
573 added to the cultures. Supernatants were stored at  $-80^\circ\text{C}$ , and HIV-1 p24 was analyzed later by  
574 ultrasensitive digital ELISA (Simoa, Quanterix) (Passaes et al., 2017).

575

#### 576 **Statistical analyses**

##### 577 *Differential gene expression*

578 For each gene, we implemented a mixed effects model to detect differential expression between cell  
579 types (Tn, Tcm, Ttm and Tem). We defined a model that included the type of cells as a fixed effect  
580 and the patient as a random effect. A p-value was then obtained by implementing a likelihood ratio  
581 test between the full model and a reduced model without the fixed effect.

##### 582 *Correlation between gene expression, metabolic parameters and HIV-1 susceptibility*

583 We computed Spearman's correlation coefficient and tested for significance.

##### 584 *Linear discriminant analysis (LDA)*

585 We implemented an LDA for each condition (control, 3d and 5d) aimed at separating the cell subsets  
586 by using the metabolic variables analyzed. The 2 first axes are represented in Figure 3.

##### 587 *Other analyses*

588 Values are presented in the graphs as medians and interquartile ranges. Statistical analyses were  
589 performed using SigmaPlot (Systat Software). Differences between CD4+ T cell subsets in different  
590 conditions were analyzed with nonparametric signed ANOVA and the multiple comparison Student-  
591 Newman-Keuls method. Differences between GFP+ and GFP- CD4+ T cells or control vs treatment  
592 culture conditions were analyzed with paired t-tests. When multiple treatment conditions were  
593 tested, ANOVA analyses and the Holm-Sidak method for multiple comparisons versus control group  
594 were used. Differences were considered significant when  $p < 0.05$ .

595

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608

#### 609 **COMPETING FINANCIAL INTERESTS**

610 The authors declare no competing financial interests.

611

#### 612 **AUTHOR CONTRIBUTIONS**

613 JCV-C, VM, MA, CP, AM, VA-F performed experiments; JCV-C, SV and AS-C analyzed the data; KB, FB,  
614 MS, M-IT and OL provided key reagents or contributed to the inclusion of study participants and the  
615 obtaining and validation of clinical information; JCV-C, NT, MS, MM-T, OL, NC and AS-C contributed  
616 to the conception and discussion of the study; JCV-C and AS-C designed the study; AS-C supervised  
617 the study; JCV-C and AS-C drafted the article; and all authors critically reviewed the manuscript.  
618  
619

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829

830



831

832 **FIGURE LEGENDS**

833 **Figure 1. CD4+ T cells subsets have different susceptibilities to HIV-1 infection**

834 Bulk CD4+ T cells from noninfected donors were infected with single-cycle HIV-1 NL4.3Δenv VSV-G  
835 particles carrying the GFP reporter gene after 5 days of suboptimal activation. A) Representative  
836 example of the proportion of GFP-expressing cells in the absence of infection (top) or 72 h after the  
837 challenge (bottom). B) Relative distribution of CD4+ T cell subsets (determined by flow cytometry) in  
838 nonactivated (NA) and activated (aCD3 5d) cells before HIV challenge and in activated cells not  
839 expressing GFP (aCD3 5d GFP-) or expressing GFP (aCD3 5d GFP+) 72 h post challenge. The pie charts  
840 (top) represent the median values (n=3 donors). The bottom panels represent the fold change in the  
841 CD4+ T cells subset contribution relative to the nonactivated condition (NA). The asterisks represent  
842 statistically significant differences (\*p<0.05; \*\* p<0.01). In a different set of experiments, CD4+ T cell  
843 subsets were flow sorted ex vivo and then cultured under nonactivated (NA) or suboptimally  
844 activated conditions for 3 (3d) or 5 days (5d) and challenged with HIV-1 NL4.3ΔenvGFP VSV-G for 72  
845 h. Flow cytometry was used to determine the % of GFP-positive cells. C) Representative example of  
846 infection levels in Tn, Tcm, Ttm and Tem cells from a donor in the different conditions analyzed. D)  
847 Medians and IQR values for experiments with cells from 6 donors. Blue bars represent Tn cells,  
848 orange bars represent Tcm cells, pink bars represent Ttm cells and violet bars represent Tem cells.  
849 Symbols represent the individual data points. Significant differences between experimental  
850 conditions are shown for each T cell subset as horizontal lines. The median infection level in NA Tn  
851 cells is displayed as a reference dashed line to facilitate comparison between T cell subsets.

852

853 **Figure 2. HIV-1 infection levels in CD4+ T cell subsets correlate with the expression levels of genes**  
854 **related to cell metabolism**

855 A) Heat maps displaying the genes differentially expressed (p<0.05) between the CD4+ T cell subsets  
856 (Tn, Tcm, Ttm and Tem) (n= 6 donors) in the absence of activation or after 3 or 5 days of suboptimal

857 activation with soluble anti-CD3 (i.e., at the time of HIV challenge). Heat maps were generated by K-  
858 means clustering (green = downregulation, red = upregulation). Data were filtered by variance  
859 ( $\delta/\delta_{\max}=0.2$ ) to reduce background noise. Gene expression data were centered to a mean value of  
860 zero and scaled to unit variance. Variables are ordered by hierarchical clustering and samples by  
861 CD4+ T cell subsets. B) Spearman's correlation between the levels of gene expression at the time of  
862 HIV-1 challenge and HIV-1 infection levels 72 h after challenge. Only significant correlations ( $p<0.05$ )  
863 are represented in the graphs (green bars). Genes highlighted in red show the group of genes that  
864 correlated with infection levels in all conditions.

865

866 **Figure 3. CD4+ T cell subsets have different metabolic profiles that coincide with their susceptibility**  
867 **to HIV-1 infection**

868 Oxygen consumption (OCR) and extracellular acidification rates (ECAR) measured before and after  
869 the addition of oligomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and  
870 rotenone/antimycin A in nonactivated (NA), 3-day activation (3d) and 5-day activation (5d) CD4+ T  
871 cell subsets. A) Median values of the metabolic variables obtained for the CD4+ T cell subsets from  
872 the 6 donors in the different conditions analyzed. B) Median and IQR basal OCR (left panel) and ECAR  
873 (right panel) and C) basal ECAR/OCR ratio for CD4+ T cell subsets in different activation states (0d, 3d,  
874 5d). Symbols represent the individual data points (n=6). Significant differences between experimental  
875 conditions are shown for each T cell subset as horizontal lines. Median values in NA Tn cells are  
876 indicated by dashed lines as a reference. Symbols represent independent experiments (n=6). D)  
877 Summary of correlations between metabolic parameters at the time of infection in NA, 3d and 5d  
878 activated CD4+ T cell subsets and the % of infected cells 72 h post infection. The green color indicates  
879  $p<0.05$ . The size of the circle represents Spearman's coefficients.

880

881 **Figure 4. HIV-1-infected CD4+ T cells are characterized by higher metabolic activity levels**

882 A) HIV-infected GFP+ (green line/symbols) and noninfected GFP- (red line/symbols) bulk CD4+ T cells  
883 from donors (n=3) were flow sorted 72 h after being challenged with HIV-1 NL4.3ΔenvGFP VSV-G.  
884 Their metabolic activity levels were quantified with a Seahorse analyzer. Mitochondrial activity (as  
885 determined by OCR) and glycolysis (as determined by ECAR) were analyzed before and after the  
886 addition of oligomycin, FCCP and rotenone/antimycin A. The bioenergetic (XF) phenotypes of GFP+  
887 and GFP- cells (right panel) were determined by the  $OCR_{(basal)}$  and  $ECAR_{(basal)}$  values. The symbols  
888 represent independent experiments (n=3 donors). B) In a different set of experiments, CD4+ T cells  
889 were flow sorted 72 h after HIV challenge based first on their activation levels (high activation,  
890 CD25+/HLA-DR+ or low activation, CD25-/HLA-DR-) and then on the level of GFP expression (GFP- or  
891 GFP+ cells). The gating strategy is shown on the left panels. Pie charts (right) represent the median  
892 (n=4 donors) distribution of the CD4+ T cell subsets (determined by flow cytometry) for each sorted  
893 cell fraction as follows: high activation and GFP+, high activation and GFP-, low activation and GFP+  
894 and low activation and GFP- (n=4). C) Representative analyses of OCR and ECAR (measured as above)  
895 for each cell fraction (left) and the median and IQR basal OCR and ECAR for 6 (high activation) and 4  
896 (low activation) donors (right).

897

898 **Figure 5. Rate of glucose uptake by CD4+ T cell subsets is associated with their susceptibility to HIV-**  
899 **1 infection**

900 CD4+ T cells activated for 5 days were incubated for 30 minutes with the glucose fluorescent  
901 analogue 2NBDG. Cells were then flow sorted based on their differentiation status (Tn or Tcm) and  
902 their rate of 2NBDG uptake. Sorted cells were then challenged with HIV-1 NL4.3ΔenvGFP VSV-G  
903 particles, and the rate of infected cells was determined as the % of cells expressing GFP 72 h post  
904 infection. A) Representative example of 2NBDG content after sorting (top panels) and the levels of  
905 GFP expression 72 h later in CD4+ T cell fractions exposed (HIV-1) or not (control) to HIV-1  
906 NL4.3ΔenvGFP VSV-G particles. B) Percentage of GFP-positive cells among CD4+ T cell fractions (Tn  
907 and Tcm sorted depending on their preinfection 2-NBDG uptake). Symbols represent individual

908 values for experiments with cells from three (Tn) and five (Tcm) donors. Medians and IQR values are  
909 represented by horizontal lines.

910

911 **Figure 6. Inhibition of cell metabolic pathways blocks HIV-1 infection of CD4+ T cells**

912 A) CD4+ T cells activated for five days were infected for 72 h with HIV-1 NL4.3 $\Delta$ envGFP VSV-G  
913 particles in the absence or presence of increasing amounts of Etomoxir (left panel), 6-diazo-5-oxo-l-  
914 norleucine (DON) (middle panel), or 2-deoxy glucose (2-DG) (right panel). Blue bars represent the  
915 relative level of infection compared to the control conditions (median and IQR, n=3 donors). Purple  
916 bars represent the number of dead cells per million cells. B) Similar experiments in which CD4+ T cells  
917 were exposed to HIV-1 particles and cultured for 72 h in glucose-containing medium in the absence  
918 or presence of 2-DG (5 mM) or in culture medium without glucose (starvation) (left) or in the  
919 absence or presence of 2-Cyano-3-(1-phenyl-1H-indol-3-yl)-2-propenoic acid (UK5099) (25  $\mu$ M)  
920 (right). C) Relative number of U5-Gag copies quantified by RT-qPCR in CD4+ T cells at 6 h, 15 h or 72 h  
921 after infection with single cycle HIV-1 particles in the absence or presence of 2-DG. Individual values  
922 (symbols), medians and IQRs (horizontal lines) for five different donors are shown. D) Infection levels  
923 and number of U5-Gag copies quantified by RT-qPCR 72 h after challenge with single cycle HIV-1  
924 particles in the absence or presence of 2-DG added at the time of challenge, 4h or 8h post challenge.  
925 Values represent the relative levels of infection compared to the control condition (median and IQR,  
926 n=3 donors). E) Changes in HIV-1 infection levels in CD4+ T cell subsets as determined by flow  
927 cytometry 72 h after the infection of bulk CD4+ T cells in the absence or in presence of 2-DG (orange  
928 symbols) or Etomoxir (beige symbols). Medians (n=7 donors) are shown. F) Percentage of HIV-1  
929 productively (left panel) or latently (right panel) infected cells 72 h after the infection of CD4+ T cells  
930 with single-round HIV-1 NL4.3HIV-1DuoFluo VSVG particles in the presence of 2-DG or etomoxir.  
931 Median and IQR values from experiments with 6 donors are shown. G) p24 production in  
932 supernatants from CD4+T cell cultures 3 and 7 days after infection with HIV-1 BaL in the absence  
933 (blue bars) or presence of 2-DG (5 mM) (orange bars). Means and standard deviations for three

934 replicates are shown at each time point for experiments done with cells from three different donors.  
935 (A-F) Horizontal lines above the graphs denote statistically significant differences in the treatment vs  
936 control conditions.

937

938 **Figure 7. Suboptimal inhibition of glucose metabolism selectively eliminates preinfected CD4+ T**  
939 **cells and inhibits HIV-1 amplification from reservoirs**

940 A) Five days after suboptimal activation, CD4+ T cells were challenged with HIV-1 NL4.3ΔenvGFP VSV-  
941 G, and 3 days later, infected GFP+ (green) or noninfected GFP- (red) cells were sorted and cultured  
942 for 48 h in the absence or presence of suboptimal amounts of 2-DG. Cell viability after the treatment  
943 was measured by flow cytometry. A) One representative example is shown. B) Relative survival of 2-  
944 DG treated cells (circles) was compared to that of nontreated cells (squares) at 24 h and 48 h. C)  
945 Changes in the CD4+ T cell subset distribution 48 h after the treatment of infected bulk CD4+ T cells  
946 with 2-DG when compared with the distribution in the control condition. Median values and IQR for  
947 experiments with cells from three donors are shown. Asterisks represent statistically significant  
948 differences (\* $p < 0.05$ ; \*\* $p < 0.01$ ). D) CD4+ T cells freshly isolated from HIV-infected individuals with  
949 suppressed viremia due to cART (n=6) were stimulated with PHA/IL-2 in the absence (blue  
950 line/symbols) or presence of 2-DG (5 mM) (orange line/symbols). HIV-1 reactivation from CD4+ T  
951 cells was evaluated by ultrasensitive quantification of p24 in culture supernatants. The reactivation  
952 kinetics are shown for each individual (left panels) (mean and SD, 3 replicates). Comparison of mean  
953 p24 values in the absence or presence of 2-DG on day 14 post stimulation is shown for all six  
954 experiments (right panel).

955

956

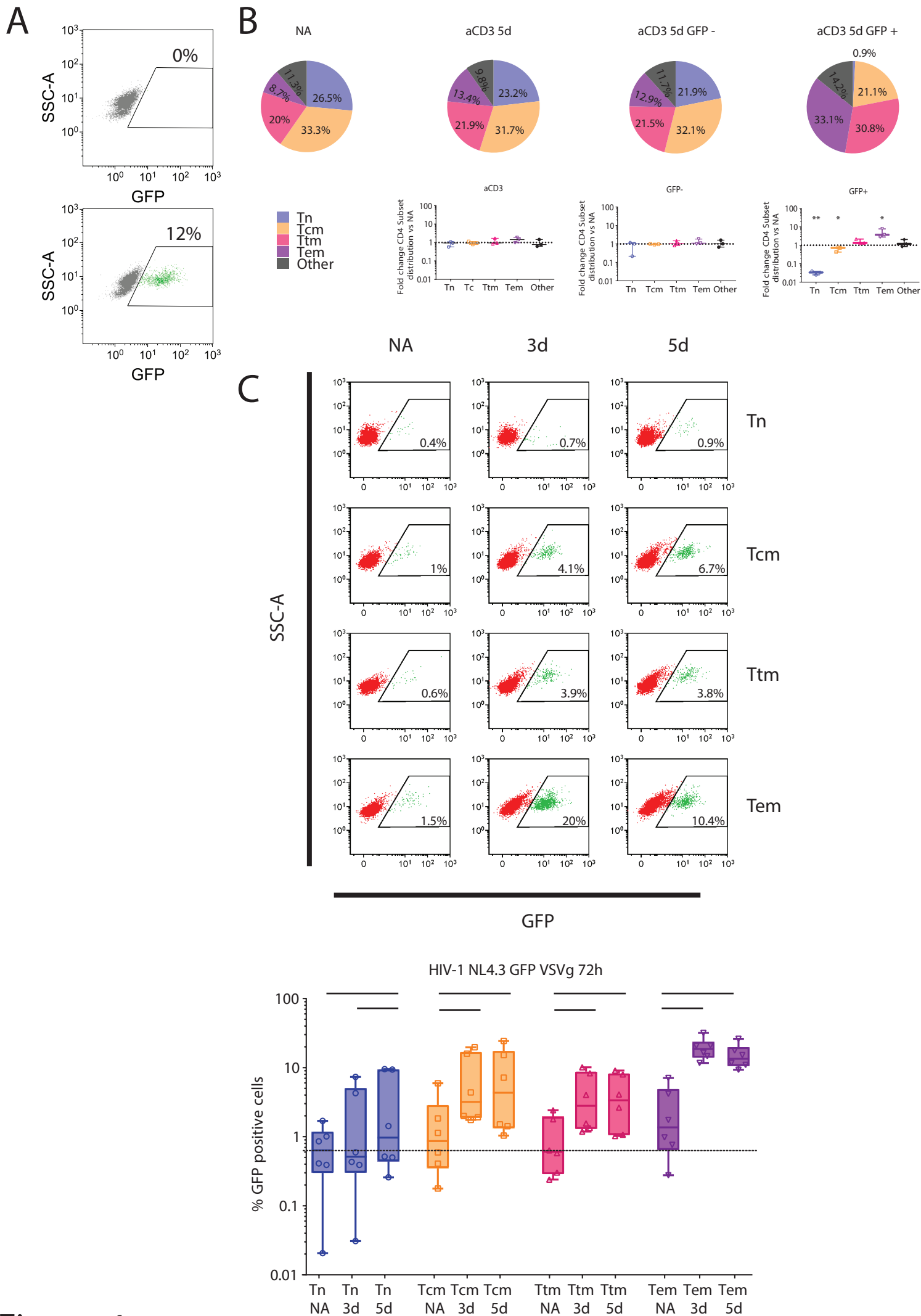


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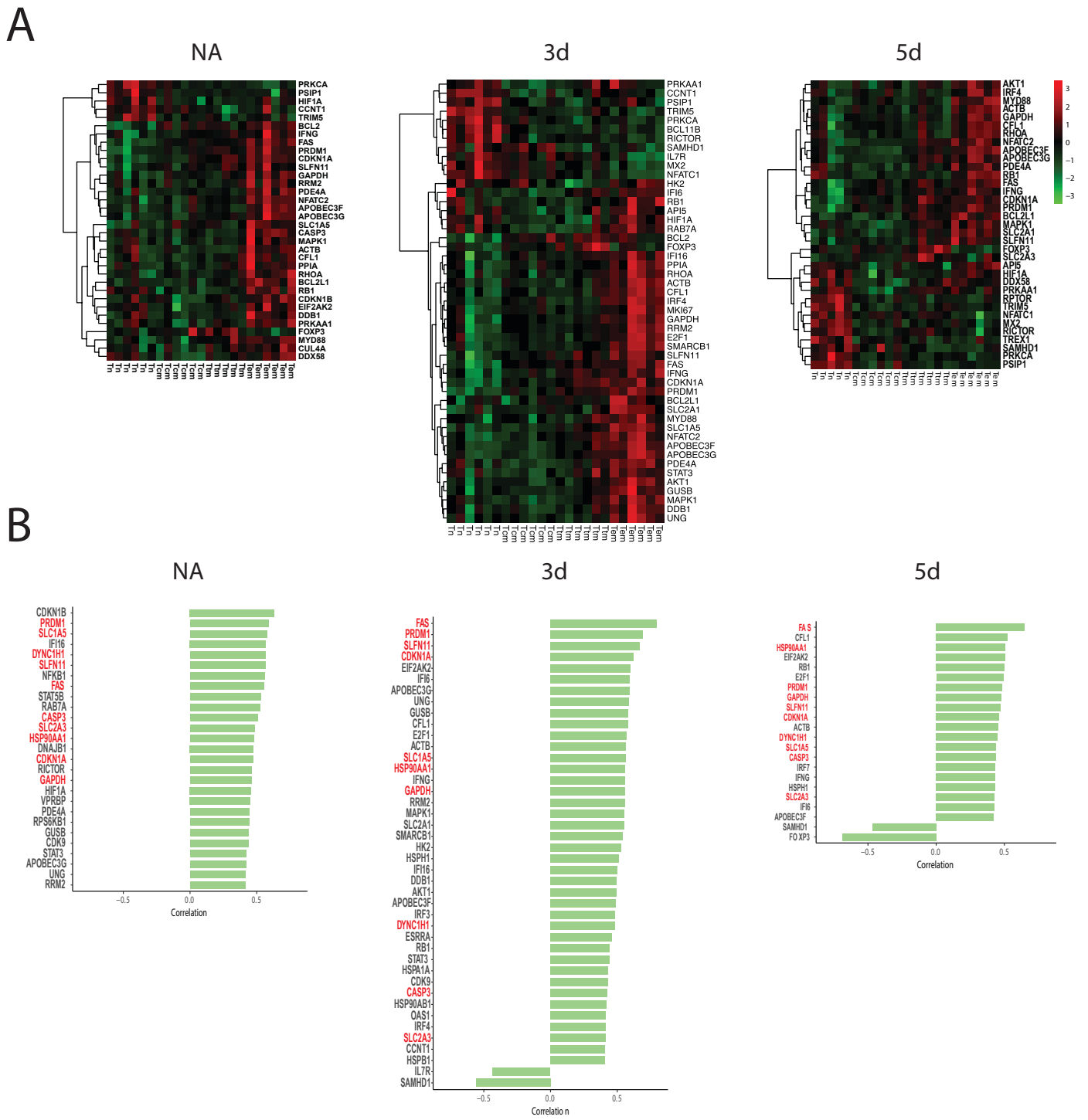
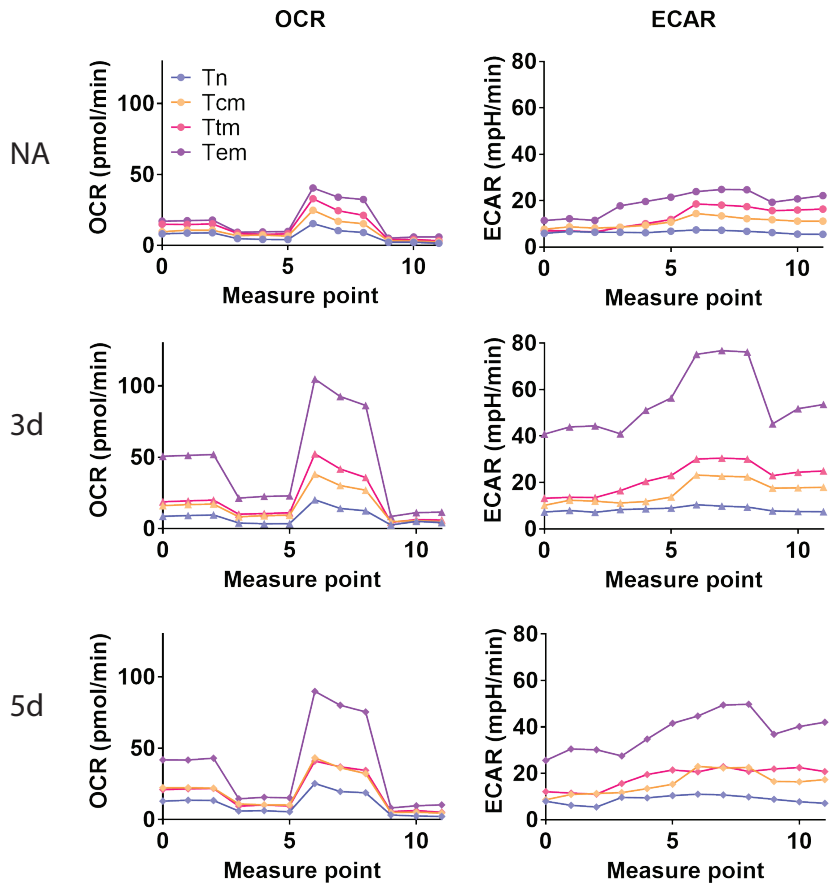
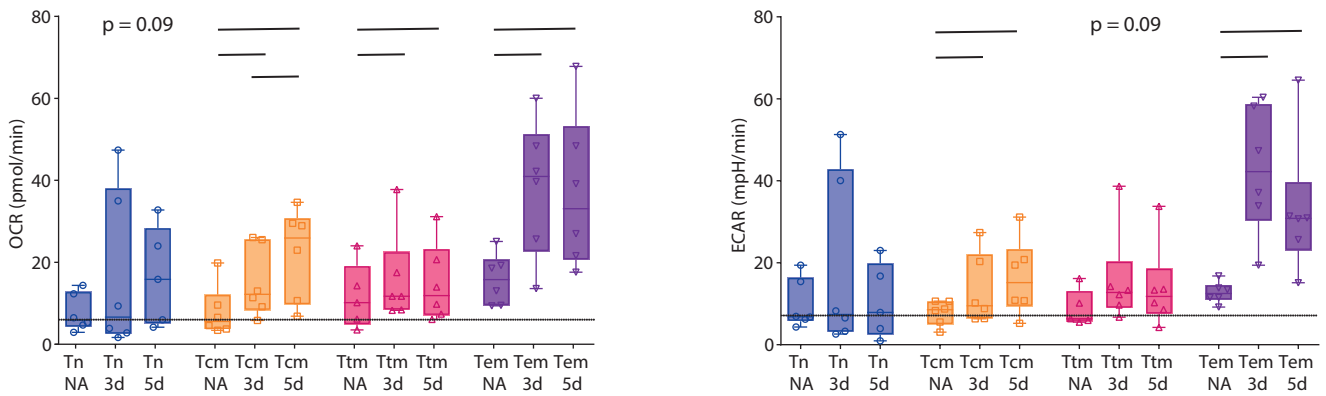


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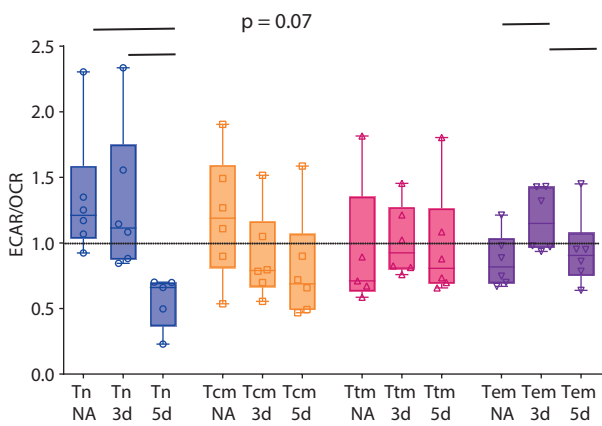
**A**



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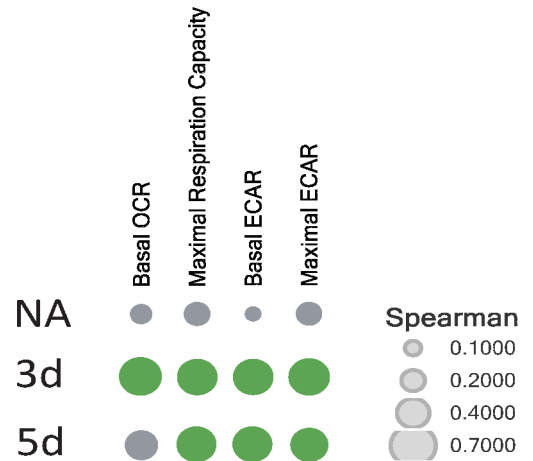


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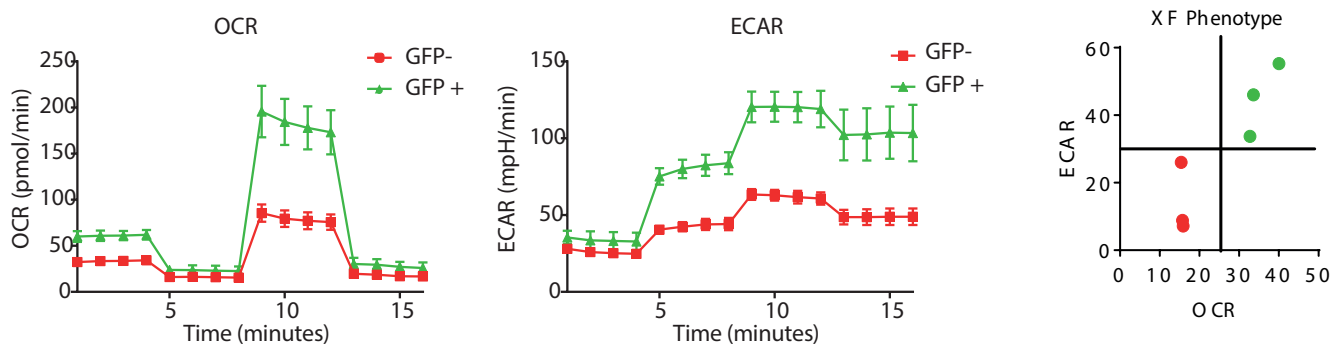
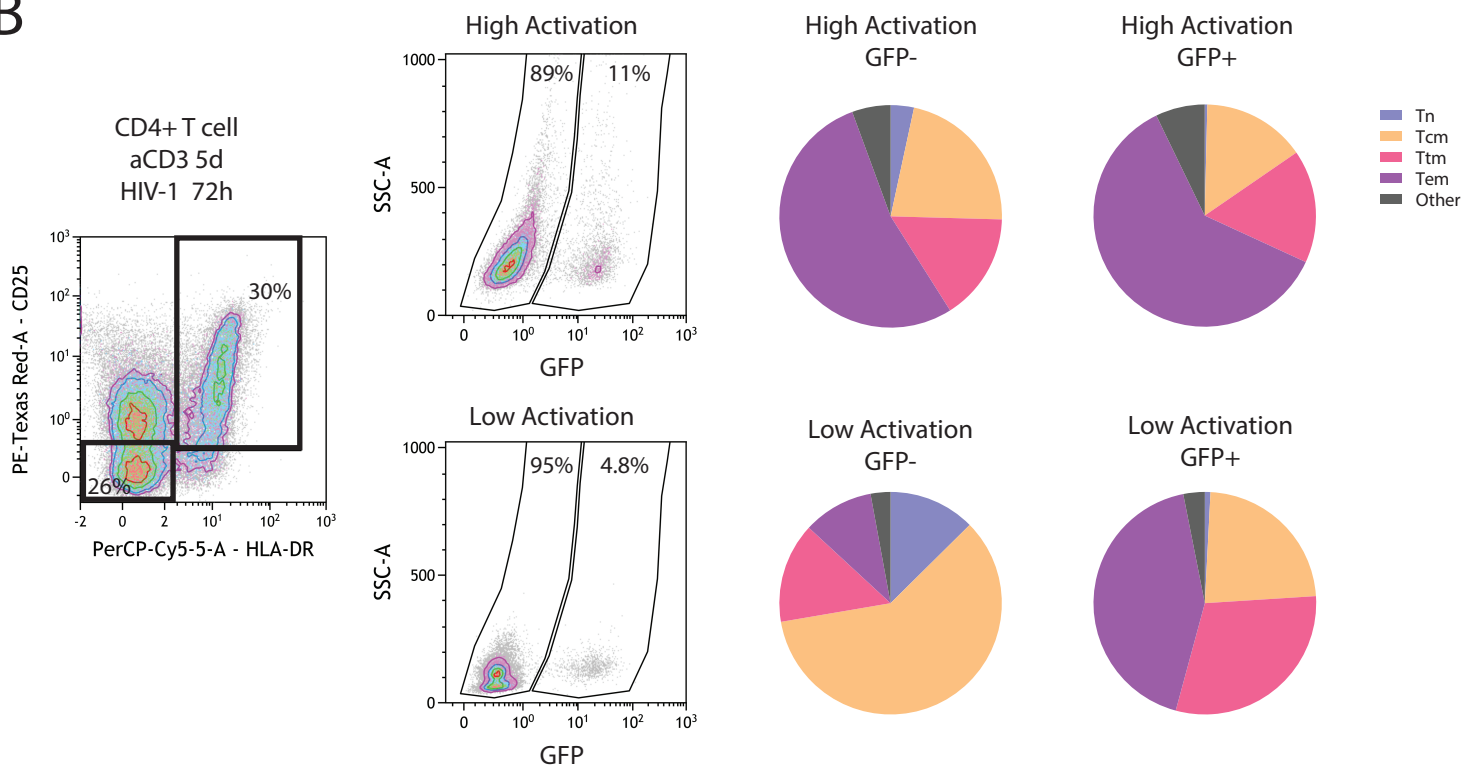
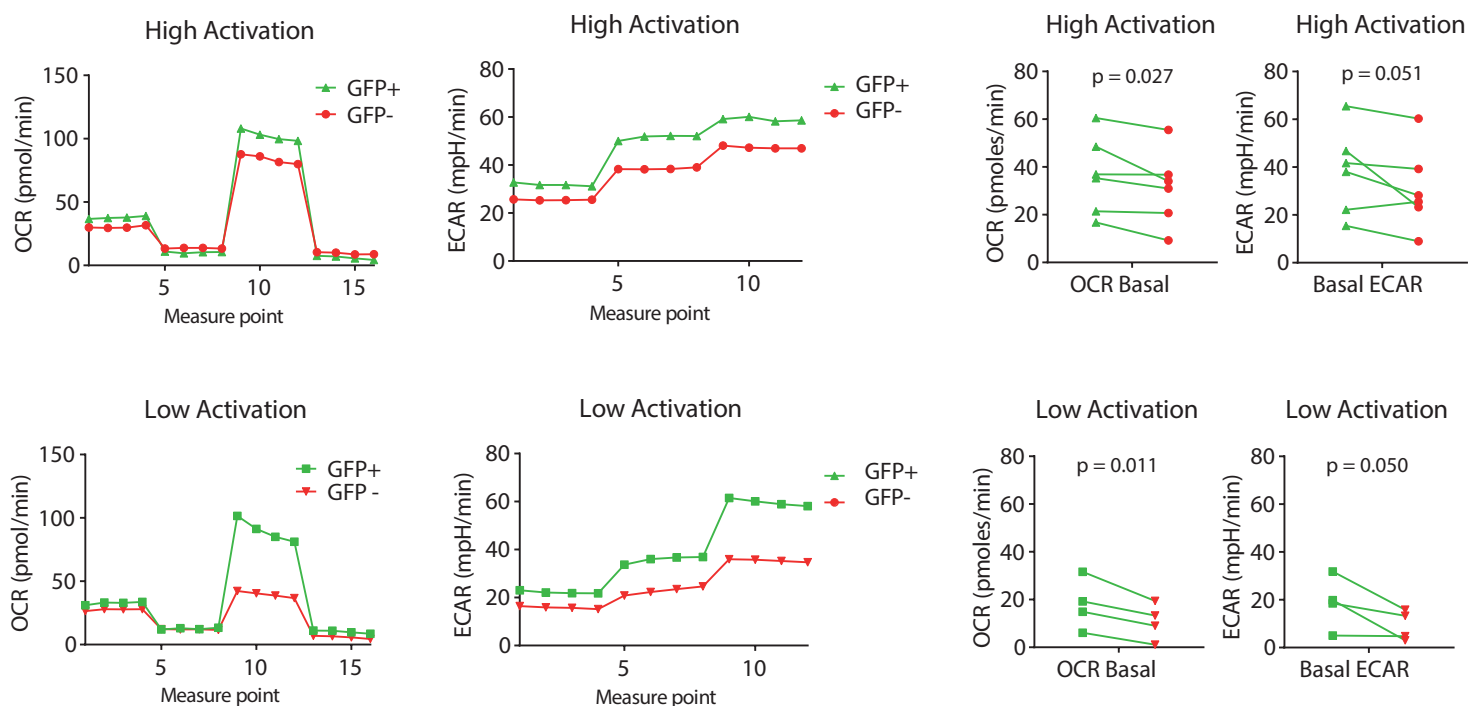
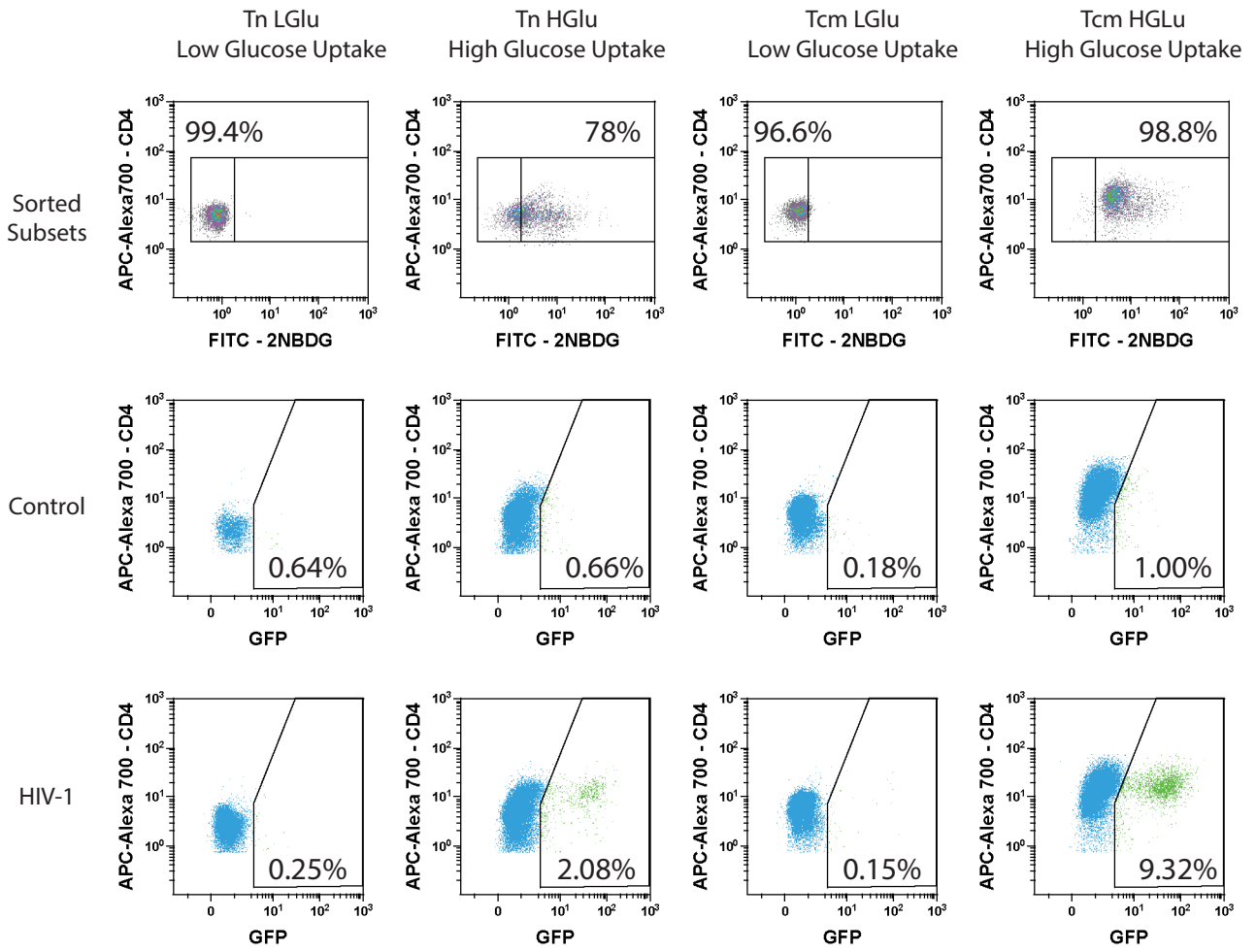
**A****B****C**

Figure 4

**A**



**B**

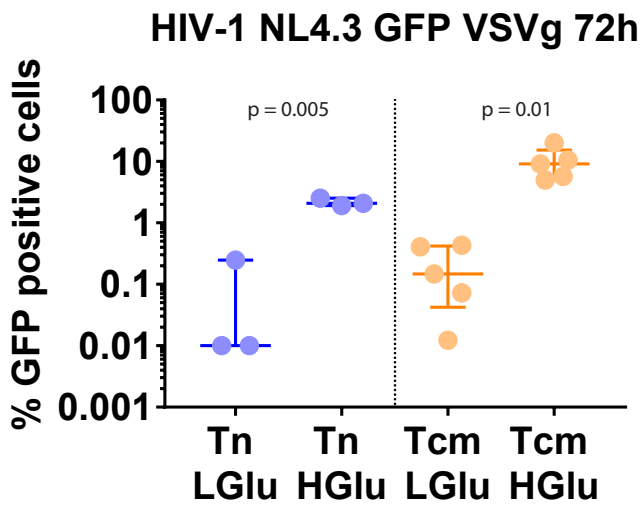
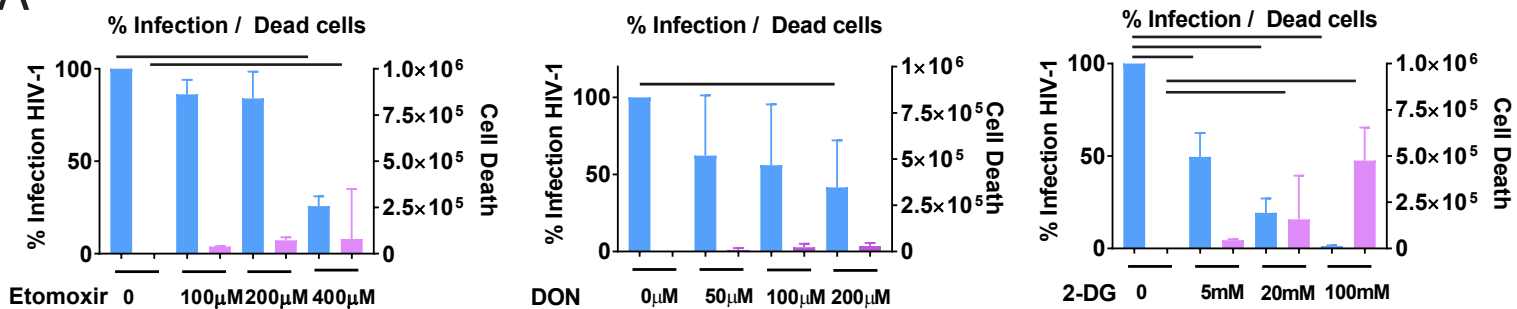
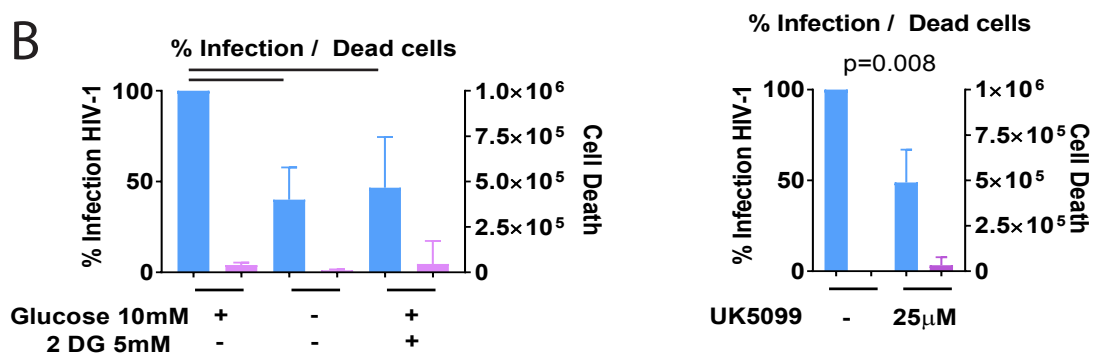


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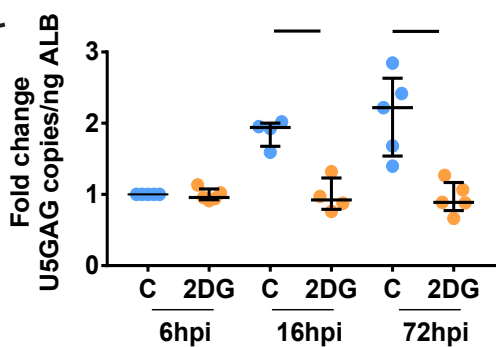
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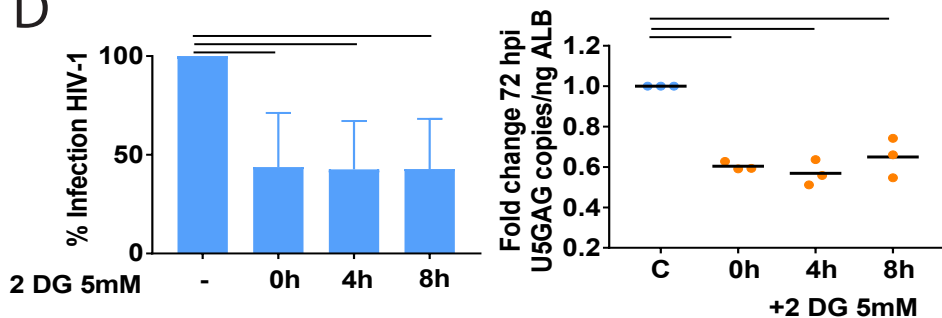
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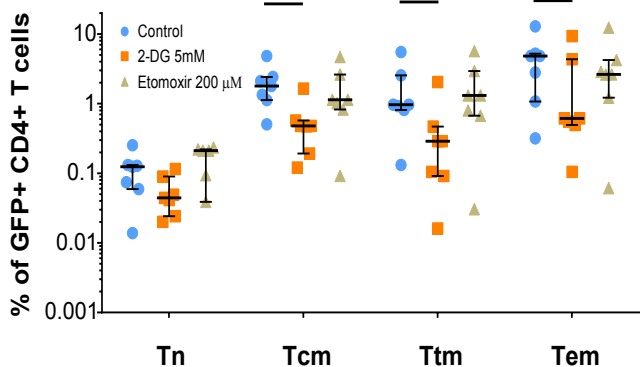
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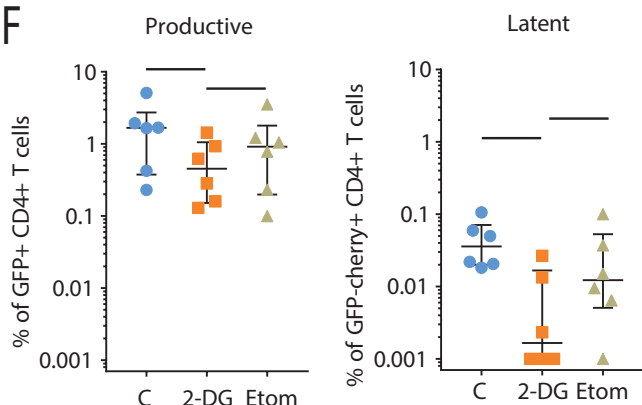
**D**



**E**



**F**



**G**

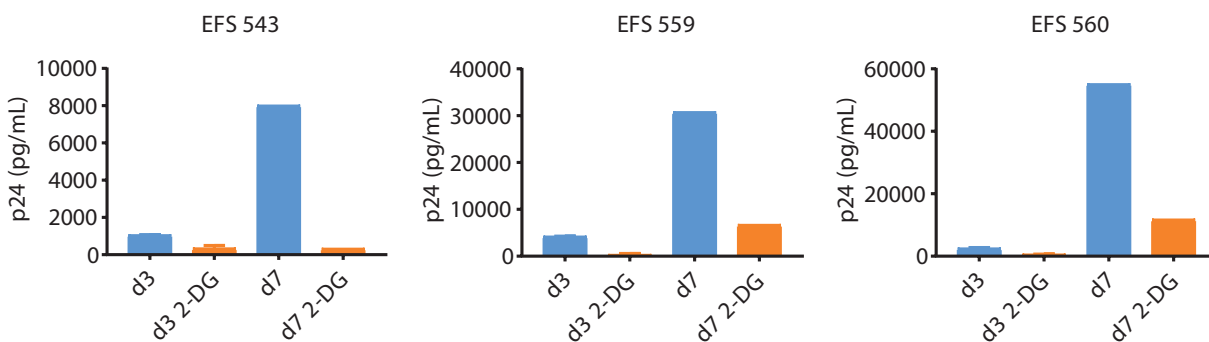


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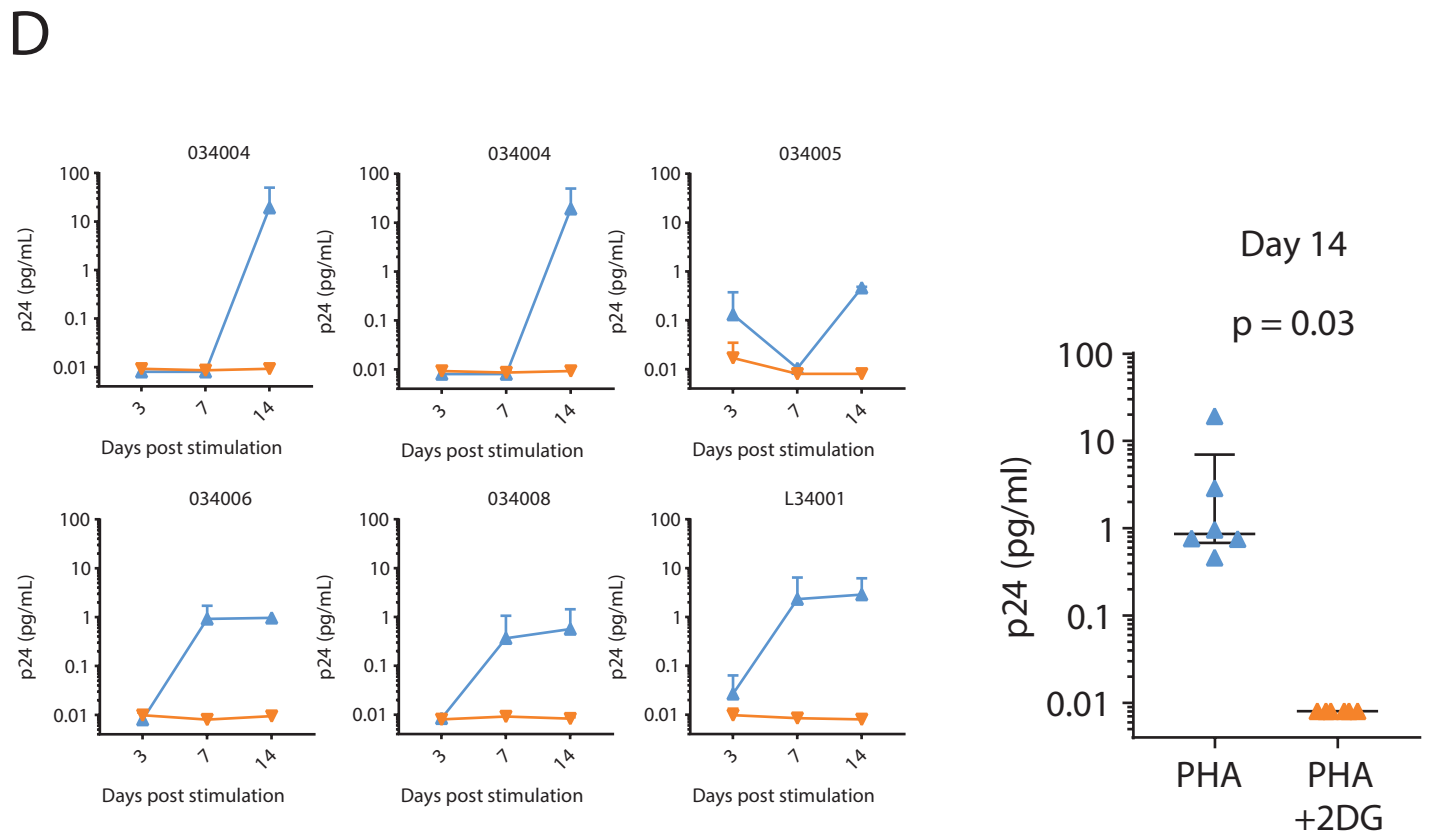
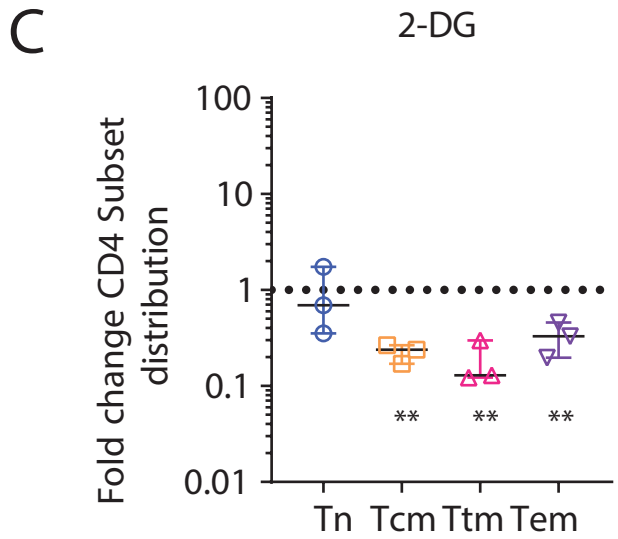
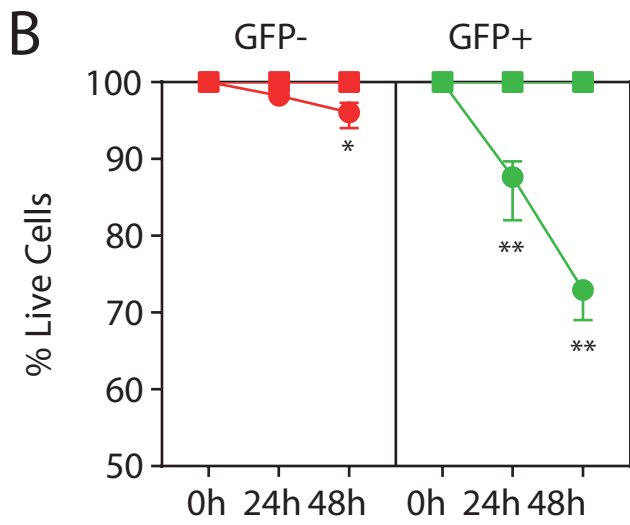
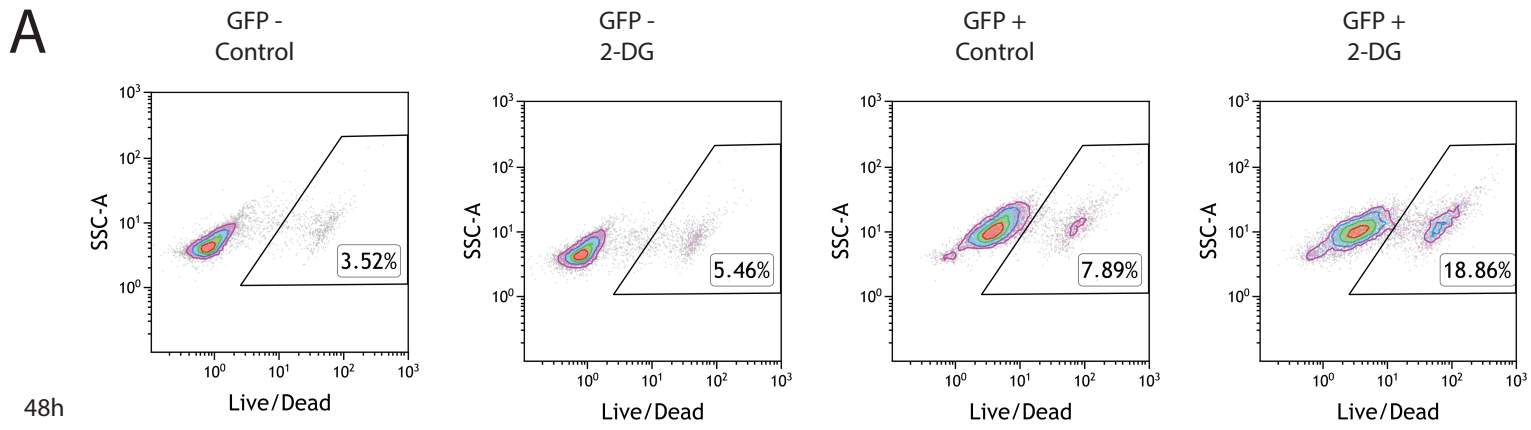


Figure 7

1 **Supplemental Material Legends**

2 **Figure S1**

3 A representative example of the flow cytometry gating strategy used to sort CD4+ T cell subsets.

4 Naïve (Tn; CD4-CD45RA+, CCR7+, CD27+, CD95-), central memory (Tcm; CD4-CD45RA-, CCR7+,  
5 CD27+), transitional memory (Ttm; CD4-CD45RA-, CCR7-, CD27+) or effector memory (Tem; CD4-  
6 CD45RA-, CCR7-, CD27-) displayed as standard pseudocolor dot plots. These sorted CD4+ T cell  
7 subsets were used in the gene expression analyses, metabolic profile assays and HIV-1 susceptibility  
8 assays. Table with gender, age and relative CD4+ T cell subset distribution ex vivo for each of the  
9 donors studied in the gene expression analyses, metabolic profile assays and HIV-1 susceptibility  
10 assays.

11

12 **Figure S2**

13 Spearman's correlation between the levels of expression of all genes analyzed at the time of HIV-1  
14 challenge and HIV-1 infection levels 72 h after challenge for nonactivated (NA), 3-day activation (3d)  
15 or 5-day activation (5d) CD4+ T cells. Significant correlations ( $p < 0.05$ ) are shown in green. Venn  
16 diagram and table showing the genes correlating with infection in the different activation conditions.

17

18 **Figure S3**

19 Representative example of flow cytometry results at the end of the glucose uptake assay showing  
20 levels of 2-NBDG in nonactivated and 5-day activation CD4+ T cells. We defined the following 3 gates  
21 based on 2-NBDG capture levels: low, medium and high (upper panels). The levels of 2-NBDG  
22 captured by the different CD4+ T cell subsets are shown in the overlapping histogram (middle  
23 panels). The relative contributions of CD4+ T cell subsets to the low, medium and high 2-NBDG cell  
24 fractions are displayed in the cumulative bar chart (lower panels).

25

26 **Figure S4**

27 A representative example of GLUT-1 surface expression (left panel) and glucose uptake (right panel)  
28 (2-NBDG assay) in the CD4+ T cell subsets studied here after 5 days of suboptimal activation. CD4+ T  
29 cells were incubated with 2-NBDG and stained with a recombinant protein that recognizes GLUT-1  
30 and set of antibodies to determine the CD4+T cell subsets.

31

### 32 **Figure S5**

33 Correlation between metabolic parameters (basal OCR, maximal respiratory capacity, basal and  
34 maximal ECAR measure with a Seahorse analyzer) at the time of infection in NA, 3d and 5d activated  
35 CD4+ T cell subsets and the % of infected cells 72 h post infection. Each symbol represents values for  
36 values for one CD4+ T cell subset from one individual. The p values of Spearman correlation analyses  
37 are shown. Lines depict linear regression of analyzed data.

38

### 39 **Figure S6**

40 A) Representative example of the flow cytometry gating strategy used to sort noninfected GFP- and  
41 infected GFP+ CD4+ T cells 72 h after challenge with single-cycle HIV-1 GFP particles and GFP  
42 expression in the purified populations. Representations are displayed as standard pseudocolor dot  
43 plots. B) Representative example of the glycolytic activity of GFP+ and GFP- sorted cells analyzed  
44 before and after the addition of glucose, oligomycin and 2-DG.

45

### 46 **Figure S7**

47 A) Percentage of cells among each CD4+ T cell subset expressing HLA-DR and CD25 five days after  
48 suboptimal activation with aCD3 (upper panel). Black horizontal lines above the symbols denote  
49 statistically significant differences between subsets. B) Representative example of expression levels  
50 of CD25 and HLA-DR for CD4+ T cell fractions sorted as high activation and GFP+, high activation and  
51 GFP-, low activation and GFP+ and low activation and GFP-. C) Changes in the relative contribution of

52 CD4+ T cell subsets to the GFP+ fractions when compared to their GFP- counterparts. Asterisks  
53 represent statistically significant differences (\*p<0.05; \*\* p<0.01).

54

55 **Figure S8**

56 A) Flow cytometry gating strategy used to sort CD4+ Tn and Tcm cells according to their glucose (2-  
57 NBDG) uptake levels and the 2-NBDG content of the CD4+ T cell fractions after sorting.  
58 Representations are displayed as standard pseudocolor dot plots. Sorted cells were used for HIV-1  
59 susceptibility assays. B) Representative example (left ) and summary (median and IQR, n=3 donors)  
60 (right) of the glycolytic activity of sorted Tcm cells with high glucose uptake (TcmHGlu) and low  
61 glucose uptake (TcmLGlu).

62

63 **Figure S9**

64 Representative example of the metabolic profile of five-day activation CD4+ T cells after being  
65 treated with increased concentrations of the metabolic inhibitors Etomoxir (top panel) and 2-DG  
66 (middle panel). OCR/ECAR basal ratio after the administration of 2-DG and Etomoxir (Lower panel).

67

68 **Figure S10**

69 A representative example of GFP and mCherry expression in noninfected CD4+ T cells or in CD4+ T  
70 cells 72 h post infection with the HIV Duo-Fluo I virus. As described in (Calvanese et al., 2013; Chavez  
71 et al., 2015), cells that expressed GFP alone or in combination with mCherry were considered  
72 productively infected (I), cells that expressed only mCherry were considered latently infected (L), and  
73 cells that lacked expression of both fluorescent markers were considered noninfected (NI). Pie chart  
74 diagrams show the median (n=5 donors) contribution of CD4+ T cell subsets to the pool of HIV-1  
75 productively infected (upper panel) or latently infected cells (lower panel) 72 h after infection of  
76 nonactivated (NA), 3-day activation (3d) or 5-day activation (5d) CD4+ T cells challenged with the  
77 HIV-1 DuoFluo I virus.

78 **Figure S11**

79 A) Relative infection levels (with respect to naive cells) in 5 days-activated CD4+ T cells from donors  
80 (n=9) 72h after challenge with HIV Bal or VSVG pseudotyped NL4.3ΔenvGFP particles. Infection levels  
81 were determined by flow cytometry quantification of intracellular p24 and GFP respectively (top  
82 panels). Changes in the distribution of cell subsets in HIV+ cells in relation to non-infected cells  
83 (bottom panels). The asterisks represent statistically significant differences (\*p<0.05; \*\* p<0.01; \*\*\*  
84 p<0.001). B) Infection levels in 5 days-activated CD4+ T cells 72 h post challenge with HIV-1 Bal in the  
85 absence or presence of 2-DG added at the time of challenge, 4h or 8h post challenge. Blue bars  
86 represent the relative level of infection (determined by intracellular p24 staining) compared to the  
87 control conditions (median and IQR, n=3 donors). Horizontal lines indicate statistical significant  
88 differences.

89

90 **Table S1**

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

92

93 **Table S2**

94 Clinical characteristics of HIV infected individuals whose CD4+ T cells were used in the study.

95

96 **Table S3**

97 Ranking of studied genes after analysis with the Normfinder algorithm (Aarhus University Hospital,  
98 Denmark). BECN1 was the most stable gene present in our panel and was used to normalize our RT-  
99 qPCR data.

100

101

102

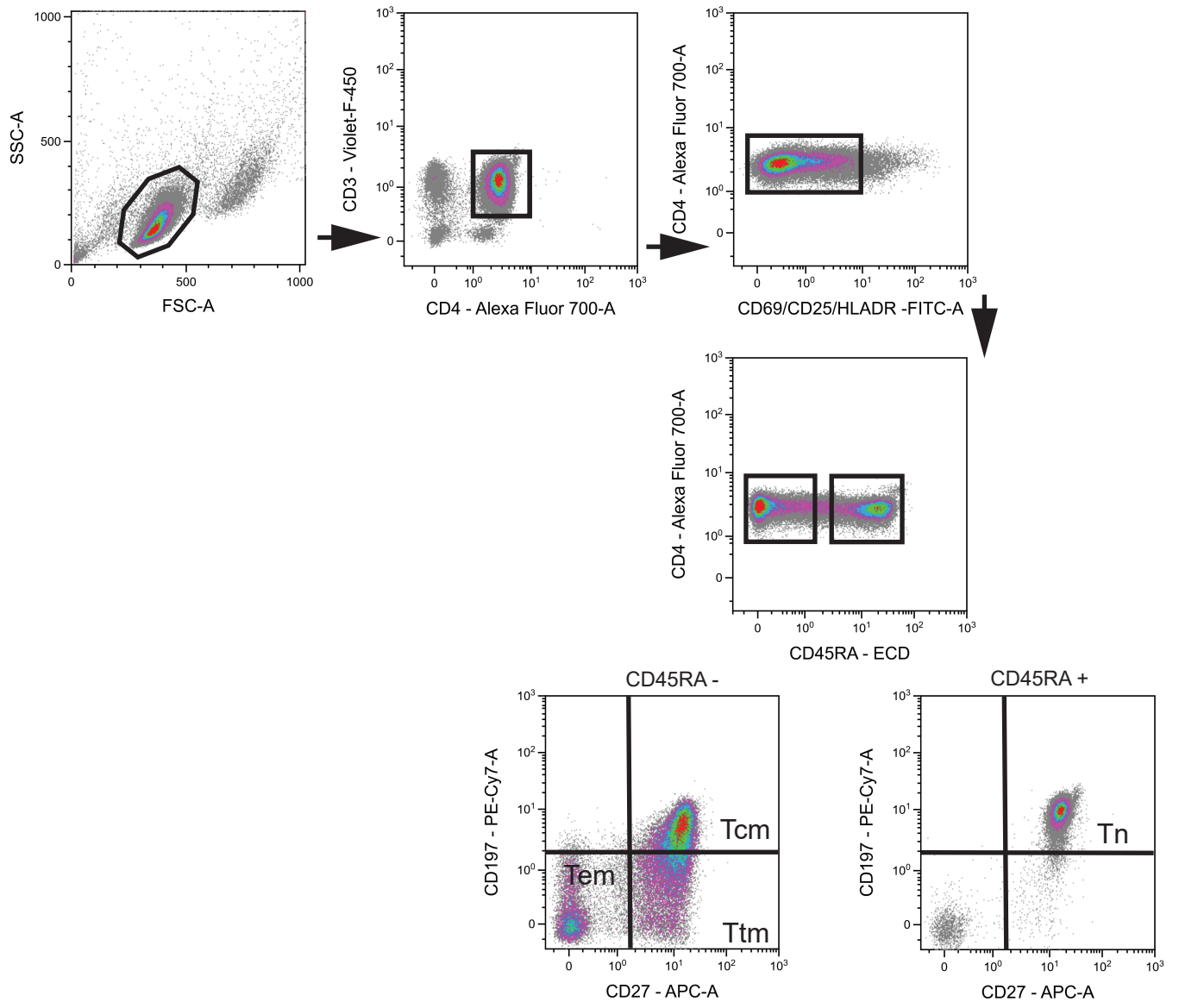


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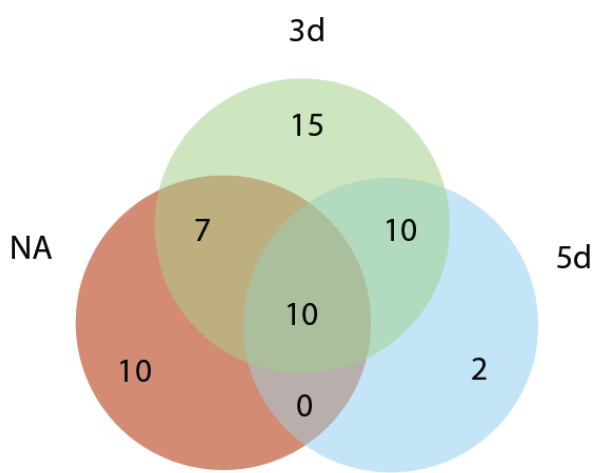
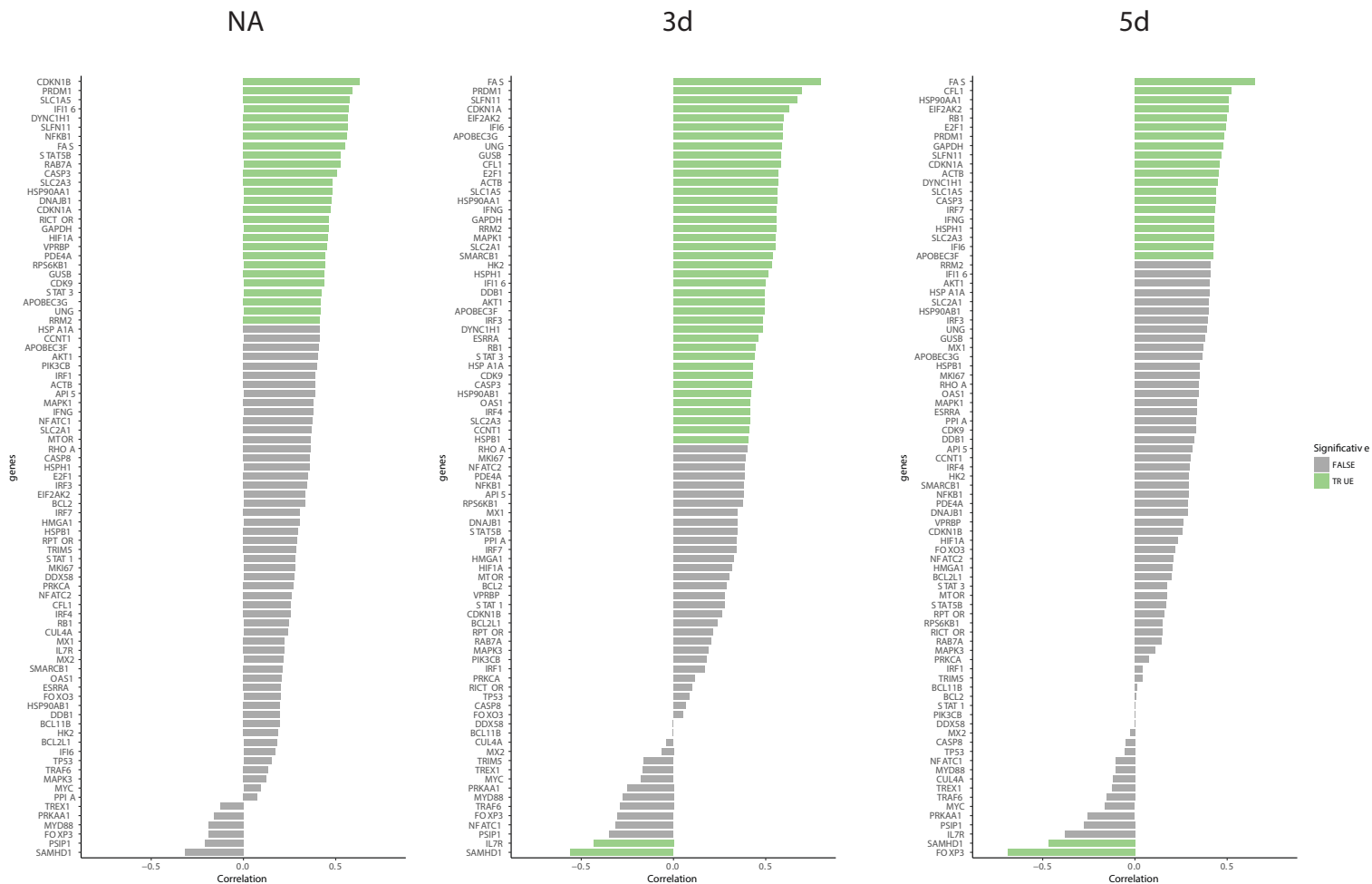
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108 Early in Both Resting and Activated Primary CD4 T Cells. *PLoS Pathog* 11, e1004955.

109



EFS#	459	466	469	470	471	472
Gender	Female	Male	Female	Female	Male	Male
Age (years)	24	18	28	51	52	36
% Tn	41.7	50.7	37	41	43.5	15.5
% Tcm	21.8	17.2	20	24.1	18.9	30.2
% Ttm	15.9	16.8	18.3	14.7	13.4	28.5
% Tem	6.89	3.04	8.11	4.79	3.42	12.9
% Others	13.71	12.26	16.59	15.41	20.78	12.9

Figure S1



NA	3d	5d	NA & 3d	3d & 5d	NA & 5d	NA & 3d & 5d
RRM2	SAMHD1	FOXP3	RRM2	SAMHD1	GAPDH	GAPDH
UNG	IL7R	SAMHD1	UNG	SLC2A3	CDKN1A	CDKN1A
APOBEC3G	HSPB1	APOBEC3F	APOBEC3G	CASP3	HSP90AA1	HSP90AA1
STAT3	CCNT1	IF16	STAT3	RB1	SLC2A3	SLC2A3
CDK9	SLC2A3	SLC2A3	CDK9	DYNC1H1	CASP3	CASP3
GUSB	IRF4	HSPH1	GUSB	APOBEC3F	FAS	FAS
RPS6KB1	OAS1	IFNG	GAPDH	HSPH1	SLFN11	SLFN11
PDE4A	HSP90AB1	IRF7	CDKN1A	GAPDH	DYNC1H1	DYNC1H1
VPRBP	CASP3	CASP3	HSP90AA1	IFNG	SLC1A5	SLC1A5
HIF1A	CDK9	SLC1A5	SLC2A3	HSP90AA1	PRDM1	PRDM1
GAPDH	HSPA1A	DYNC1H1	CASP3	SLC1A5		
RICTOR	STAT3	ACTB	FAS	ACTB		
CDKN1A	RB1	CDKN1A	SLFN11	E2F1		
DJB1	ESRRA	SLFN11	DYNC1H1	CFL1		
HSP90AA1	DYNC1H1	GAPDH	IF16	IF16		
SLC2A3	IRF3	PRDM1	SLC1A5	EIF2AK2		
CASP3	APOBEC3F	E2F1	PRDM1	CDKN1A		
RAB7A	AKT1	RB1		SLFN11		
STAT5B	DDB1	EIF2AK2		PRDM1		
FAS	IF16	HSP90AA1		FAS		
NFKB1	HSPH1	CFL1				
SLFN11	HK2	FAS				
DYNC1H1	SMARCB1					
IF16	SLC2A1					
SLC1A5	MAPK1					
PRDM1	RRM2					
CDKN1B	GAPDH					
	IFNG					
	HSP90AA1					
	SLC1A5					
	ACTB					
	E2F1					
	CFL1					
	GUSB					
	UNG					
	APOBEC3G					
	IF16					
	EIF2AK2					
	CDKN1A					
	SLFN11					
	PRDM1					
	FAS					
27	42	22	17	20	10	10

Figure S2

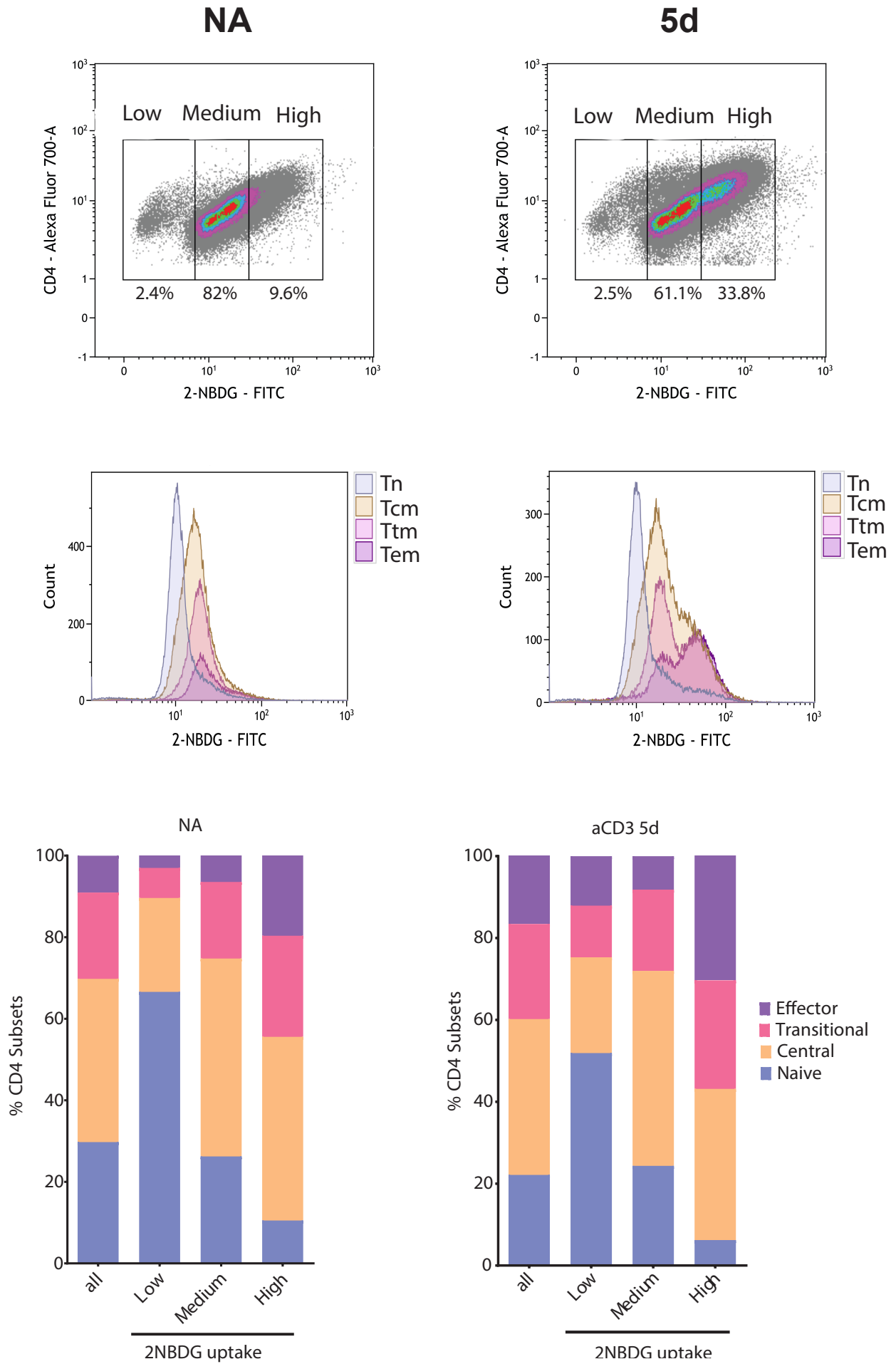


Figure S3

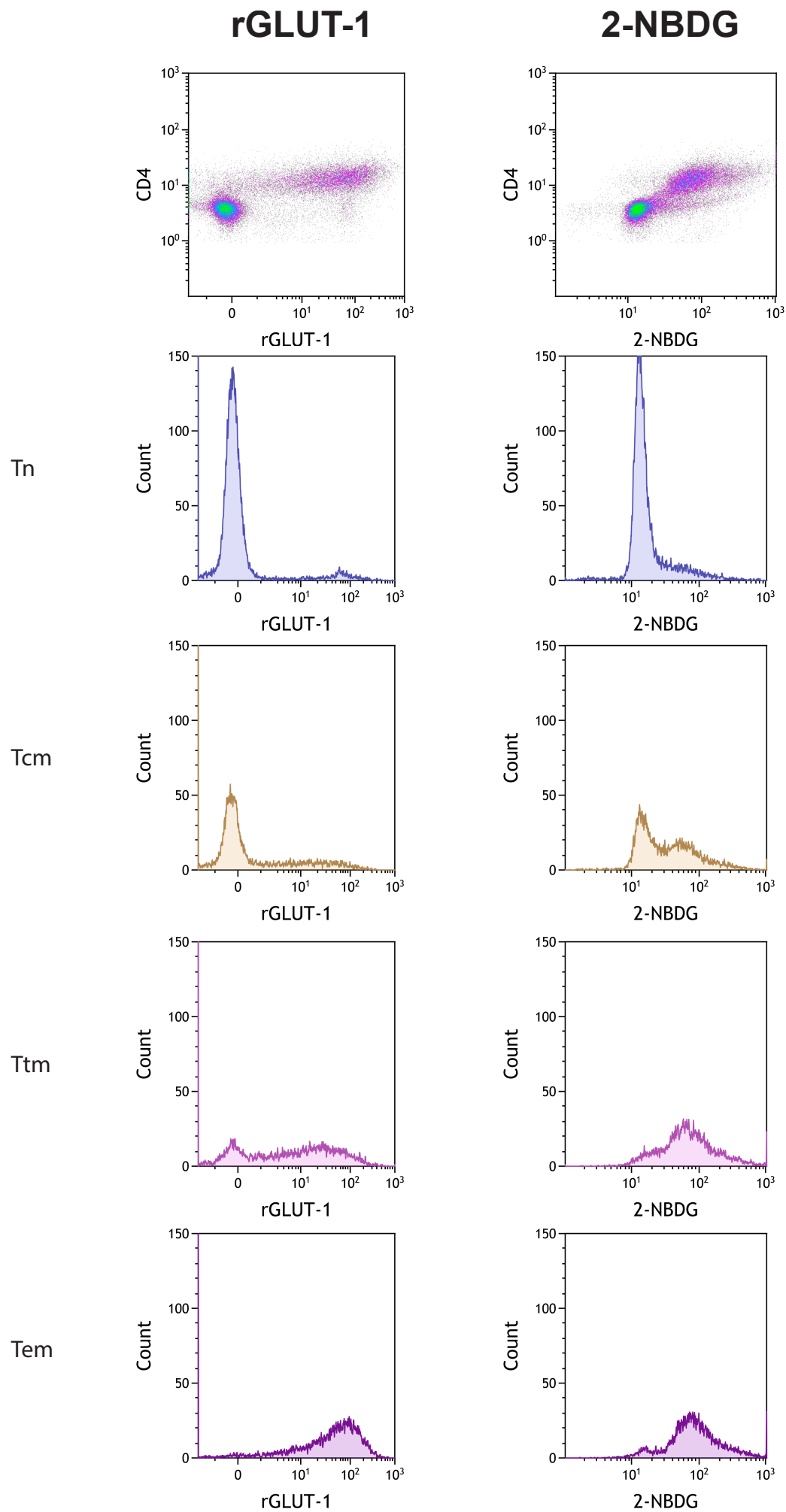


Figure S4

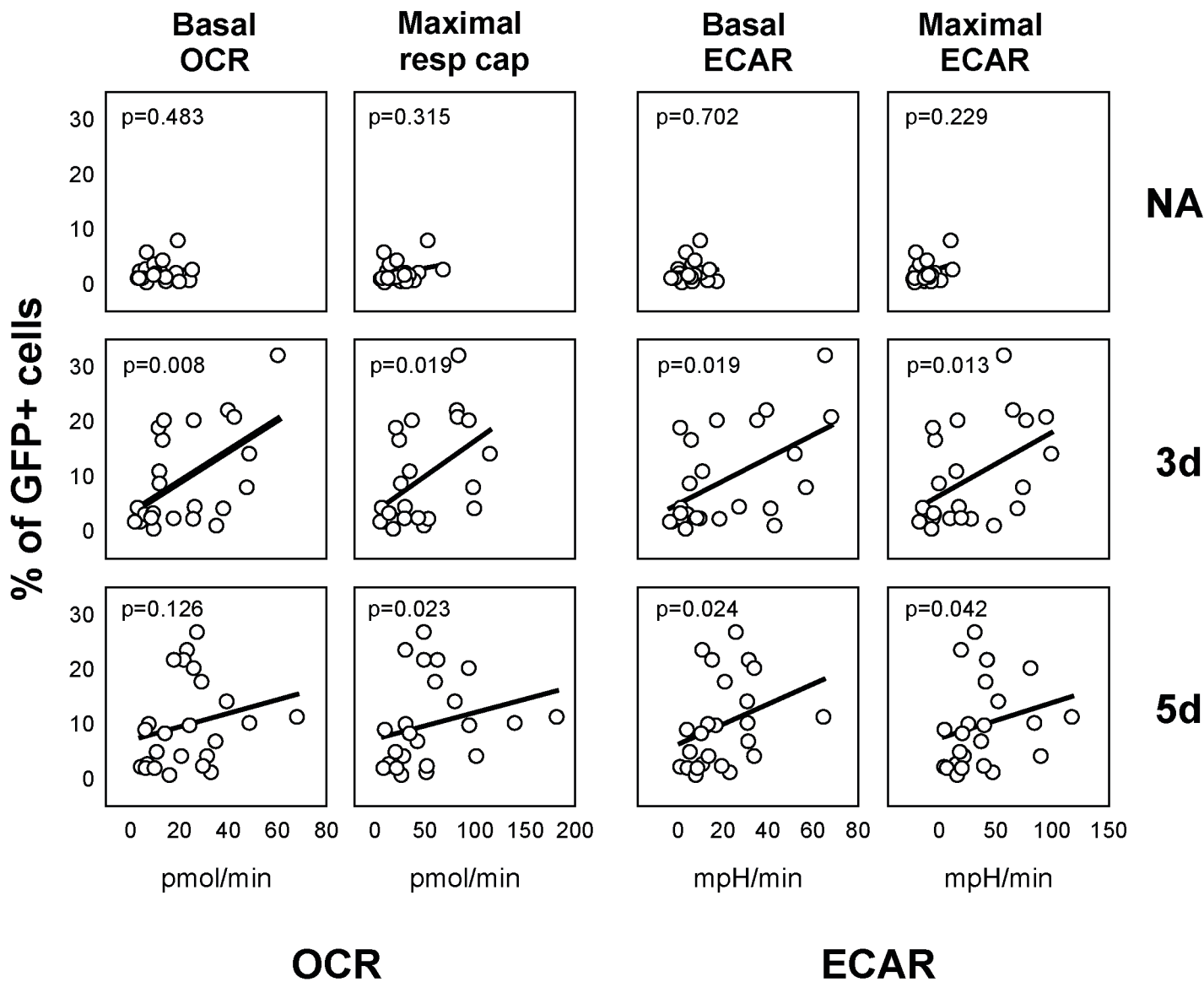


Figure S5

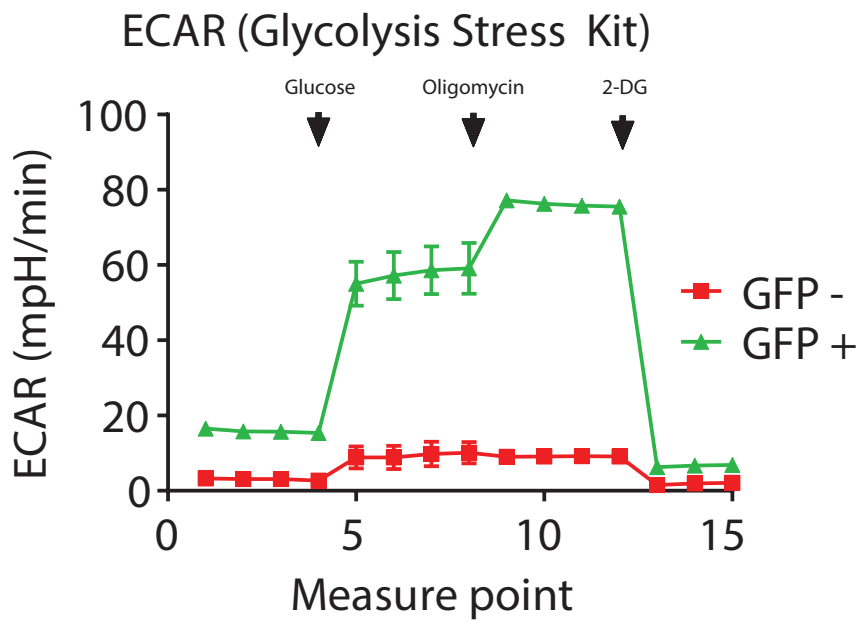
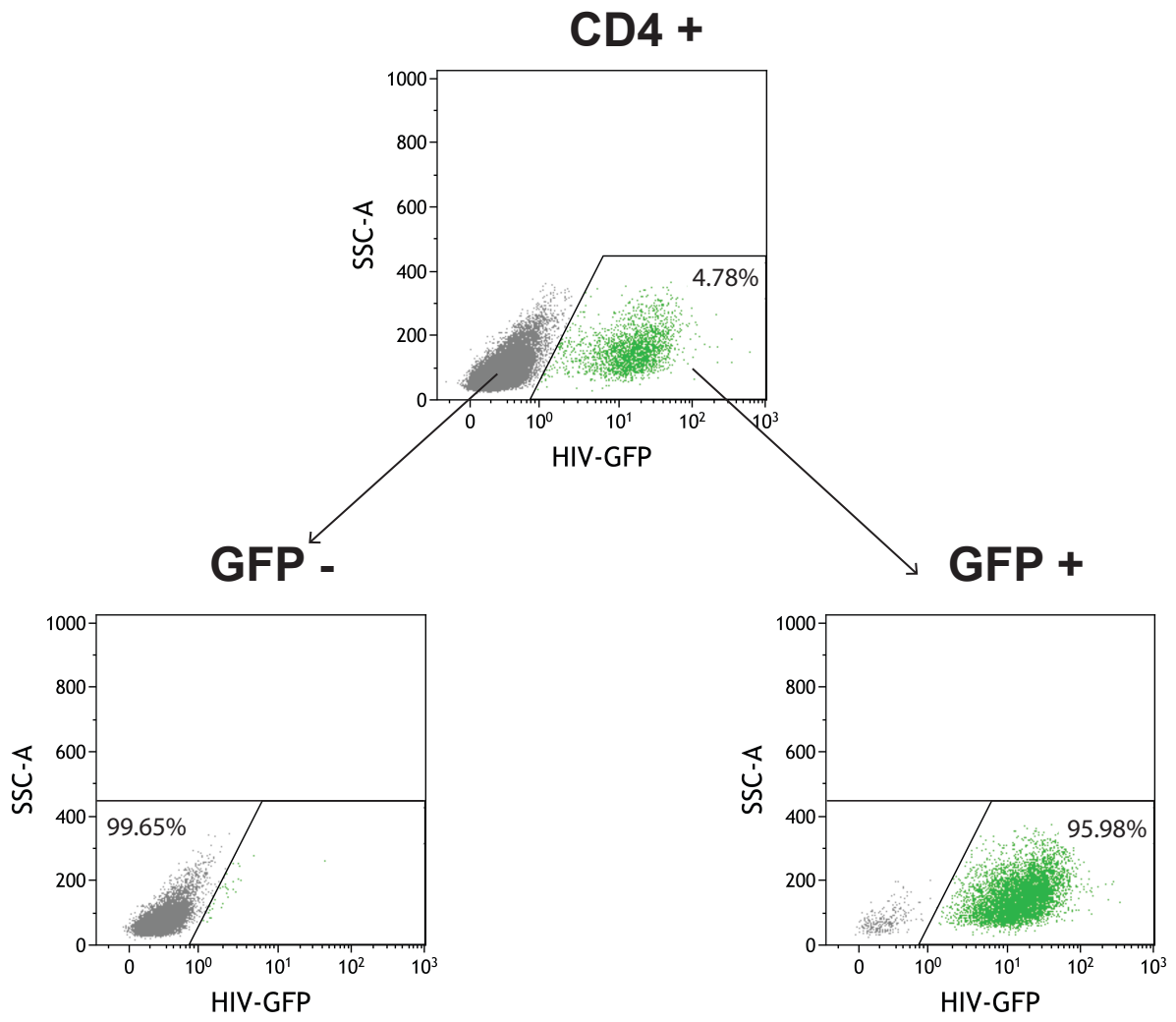


Figure S6

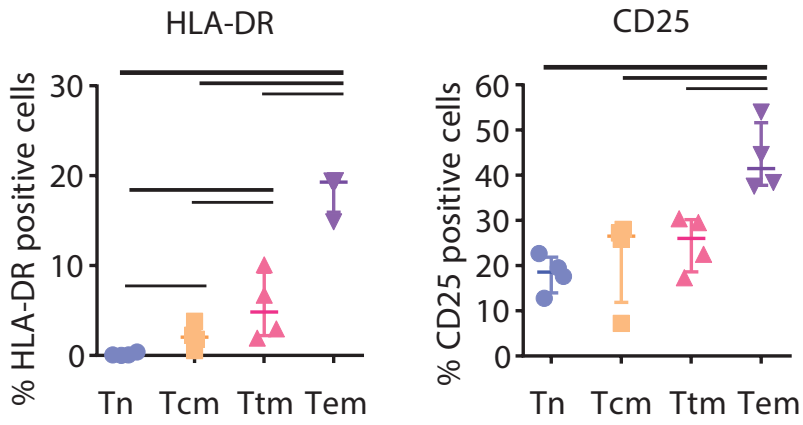
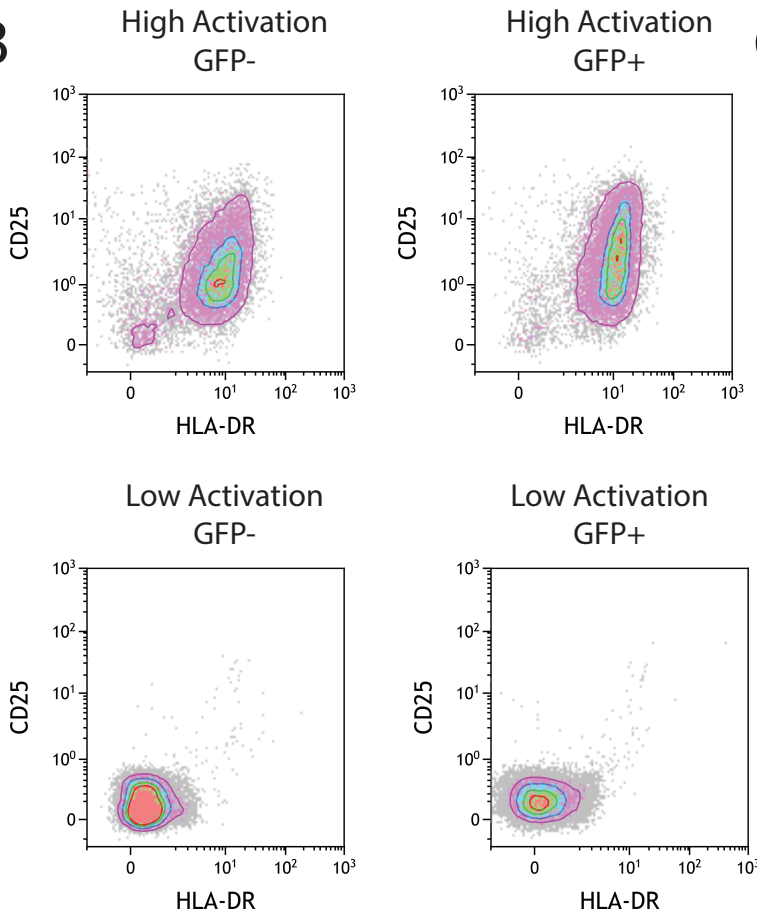
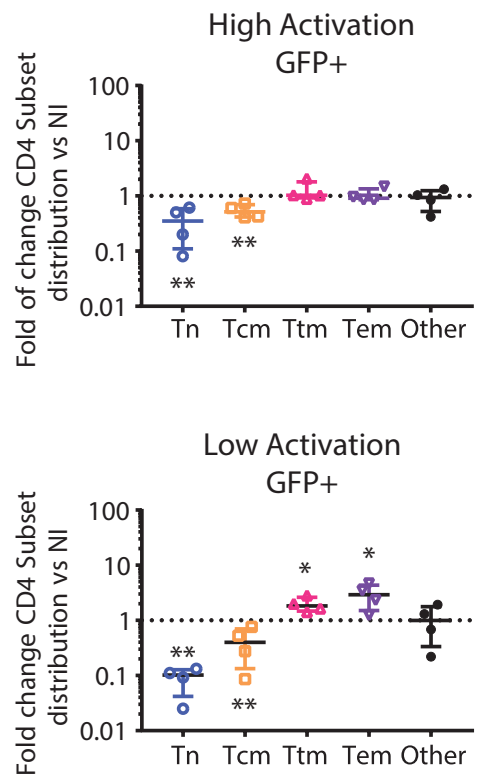
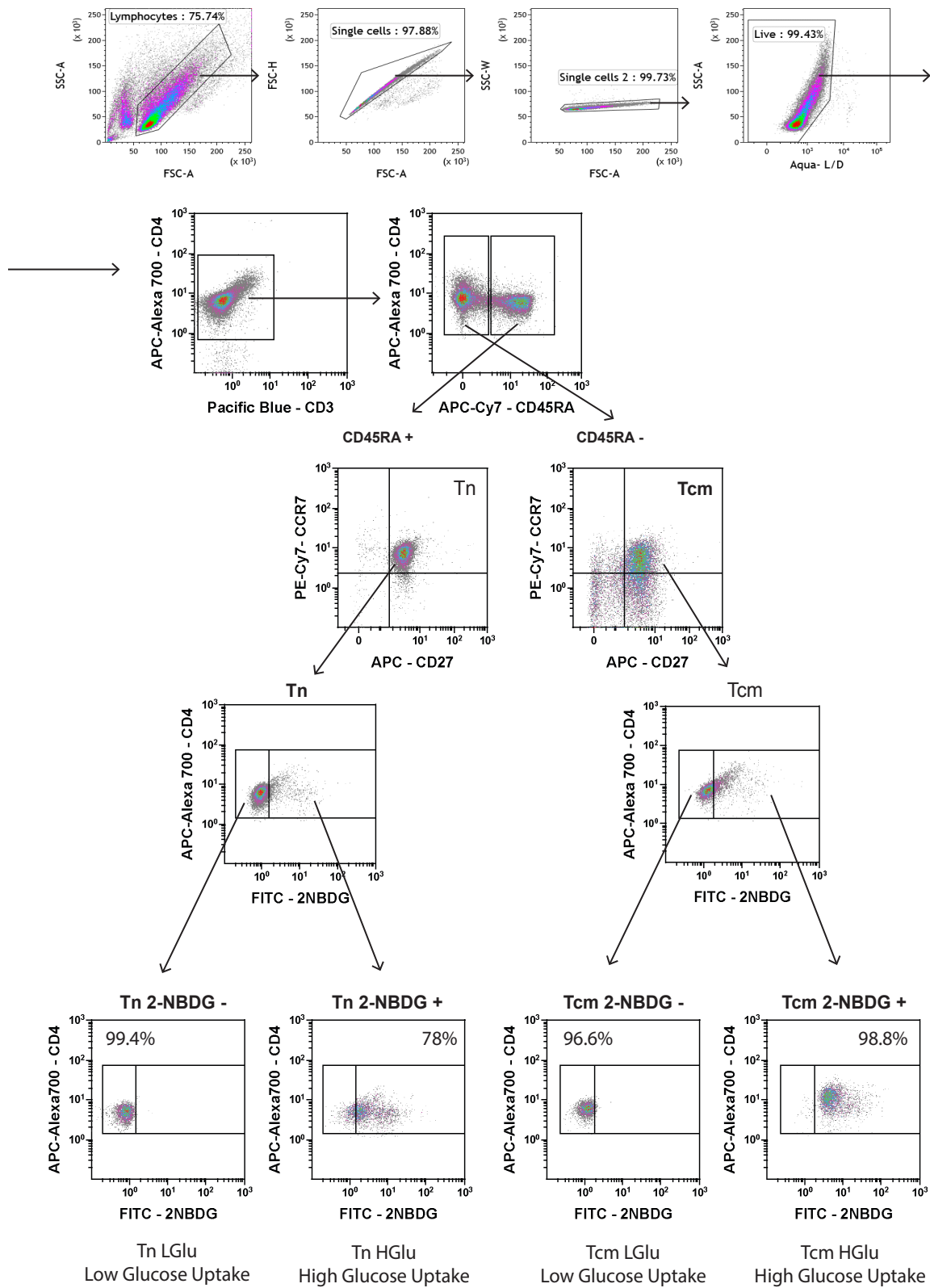
**A****B****C**

Figure S7



**A**



**B**

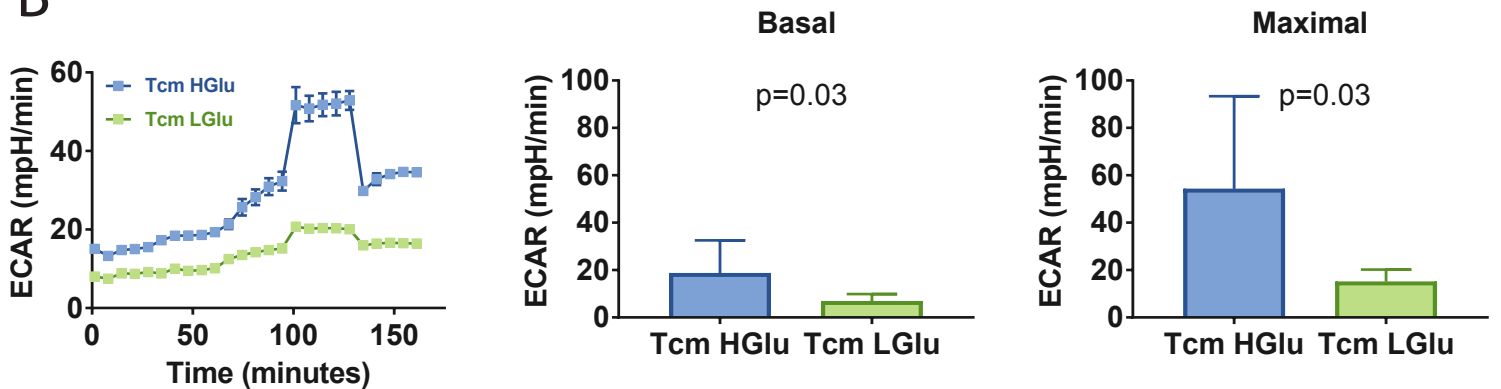
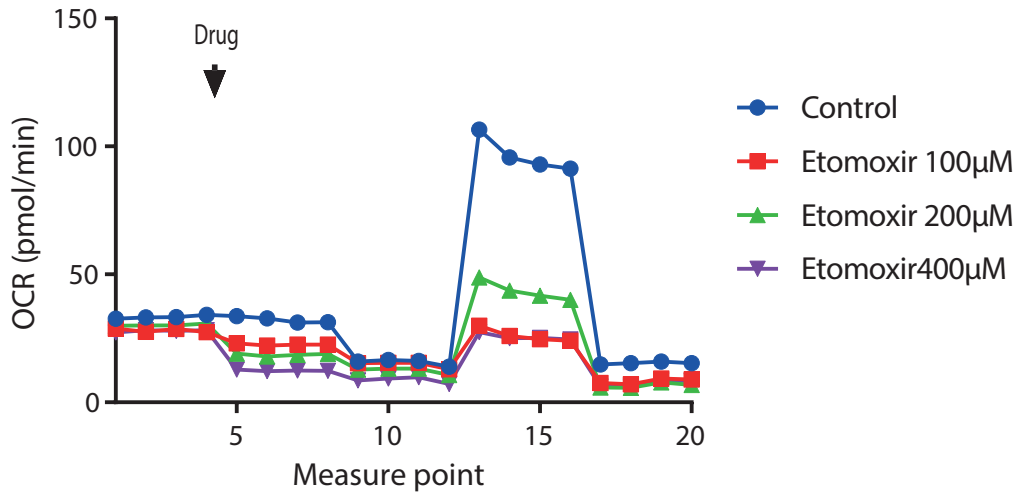
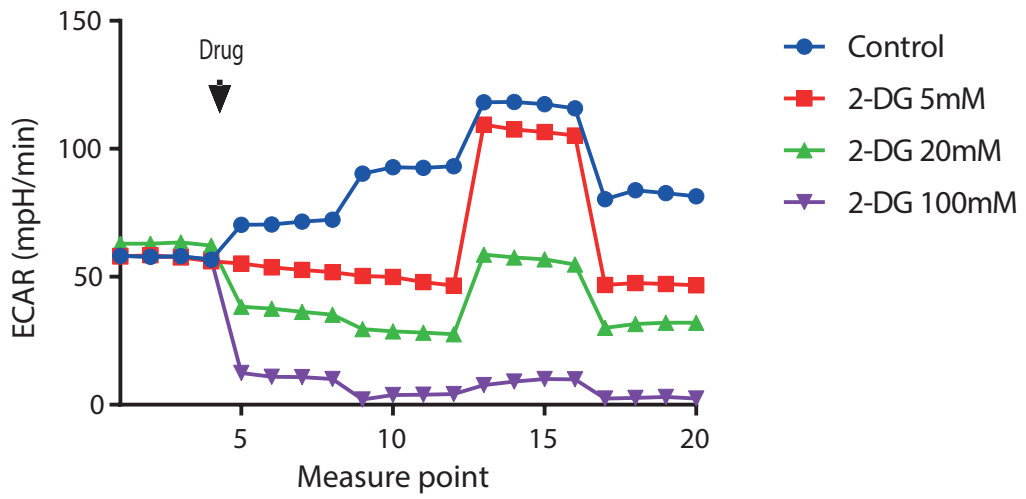


Figure S8

### Dose - Response (Etomoxir)



### Dose - Response (2-DG)



### OCR/ECAR

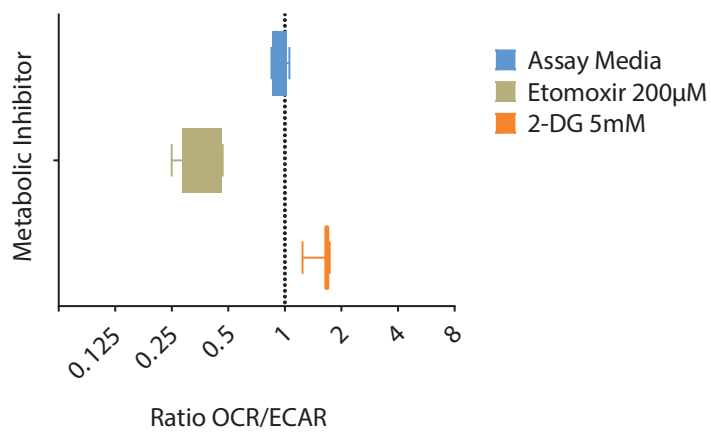
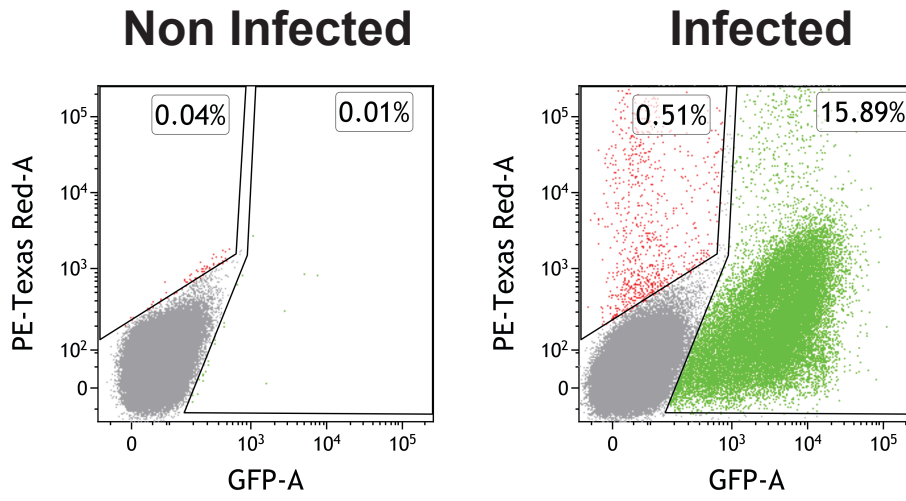


Figure S9

A



B

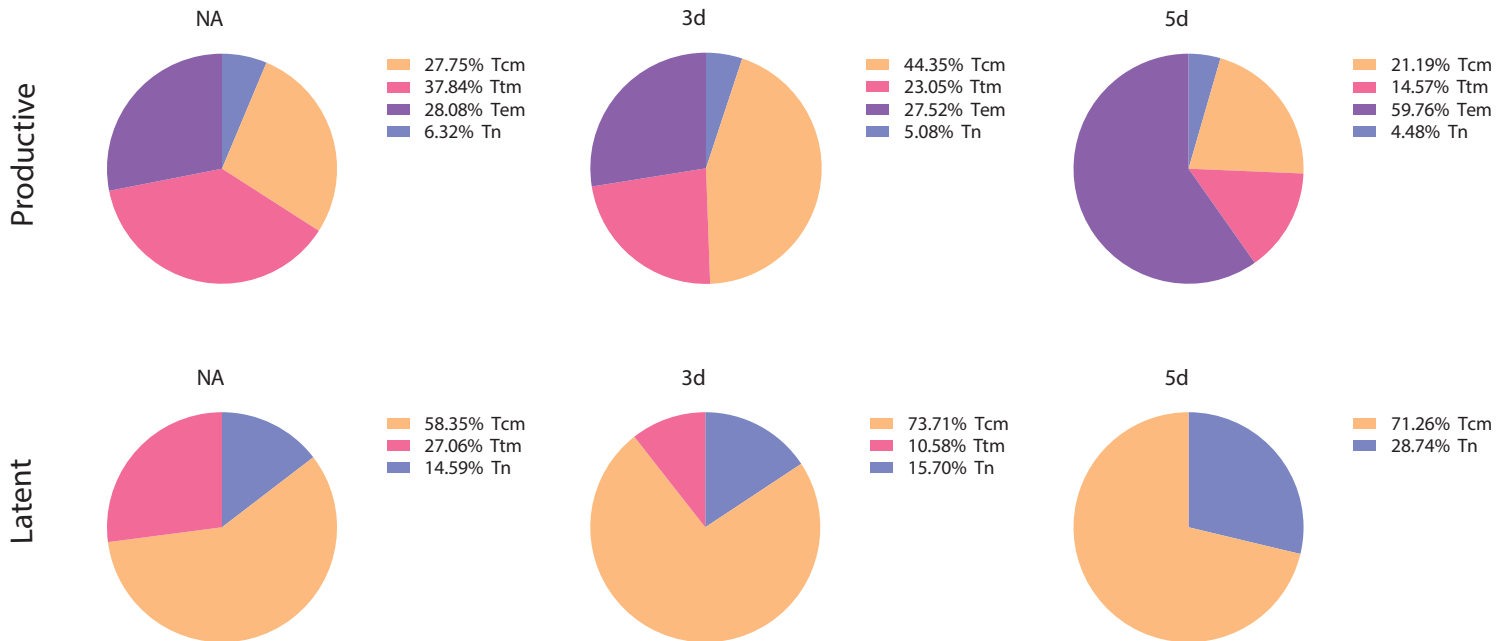
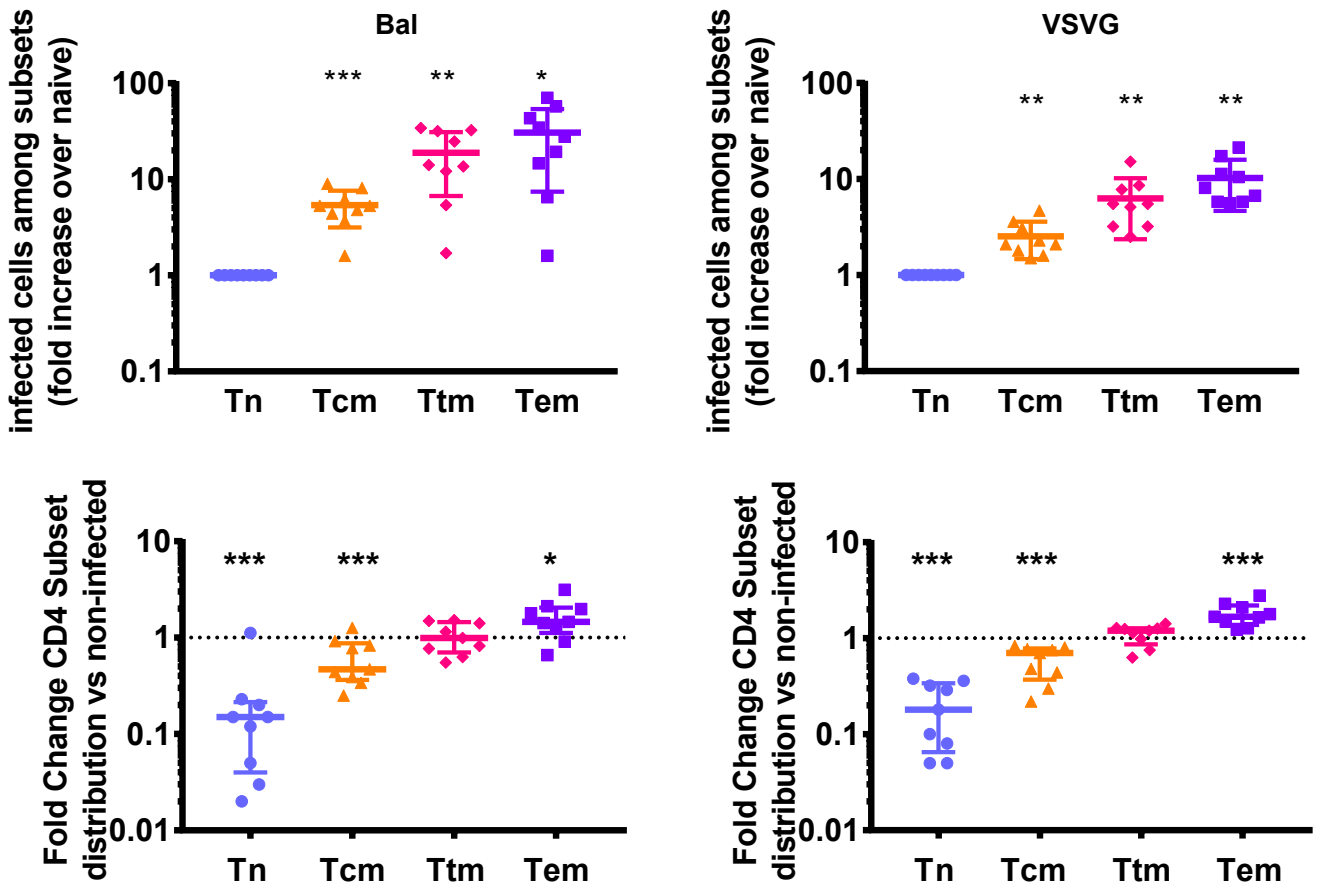


Figure S10

A



B

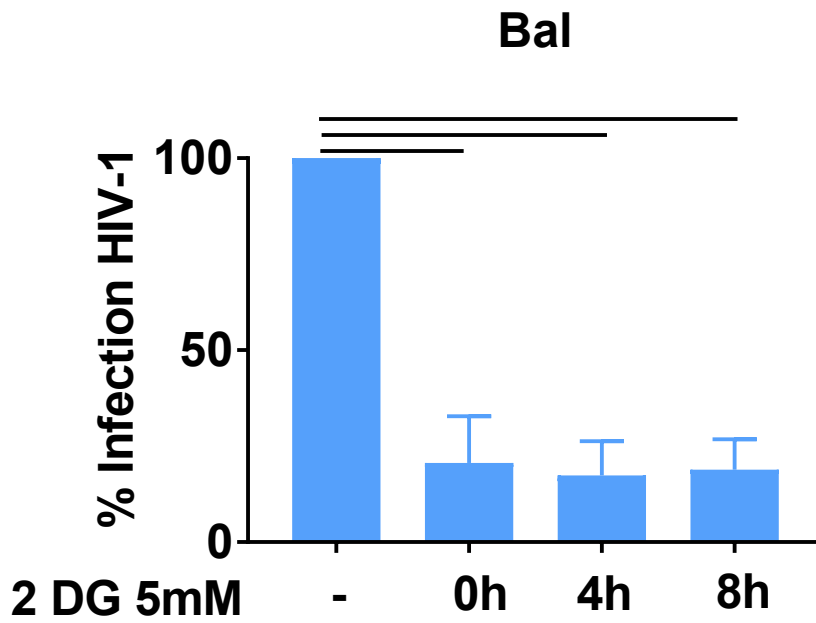


Figure S11

Gene	Design RefSeq	FP	RP	Gene Full Name
ACTB	NM_001011.3	CCAACCCGAGAGATGAC	TAGCACAGCTGGATAGCAA	actin, beta
AKT1	NM_005163.2	CACACACTACCAGAAC	TCGTGGGTCTGAAAGAGTA	v-akt murine thymoma viral oncogene homolog 1
API5	NM_006595.3	AGTTGGCCGAAACTTCCA	AGGCCCTGTGCAAGATAC	apoptosis inhibitor 5
BCL11B	NM_138576.2	CAACCCGACACTTGTCTC	CCTGCTCTTCTGAGGATGG	B-cell CLL/lymphoma 11B (zinc finger protein)
BCL2	NM_000633.2	ATGTGTGTGGAGCGTCAA	GTGCCGGTTCAGTACTCA	B-cell CLL/lymphoma 2
BCL2L1	NM_138578.1	CTGCTGCTTTGCCTAA	CCAAAACACTGCTCACTCA	BCL2-like 1
BECN1	NM_003766.3	GGTTGAGAAAGCCGAGACAC	ACTGCTCTGTGCTTCAA	beclin 1, autophagy related
CASP3	NM_004346.3	AGGCCGACTTCTGTATGCA	AACCAGGAGCCATCTTTGAA	caspace 3, apoptosis-related cysteine peptidase
CASP8	NM_001080124.1	GGAAATCTCCAAATGCAAACTGG	CAGGATGACCCCTCTTCCAT	caspace 8, apoptosis-related cysteine peptidase
CCNT1	NM_001240.3	GATTCACTGCTTCCACAGTCC	TTTTGGGCTGCTCTCCA	cyclin 11
CDK9	NM_001261.3	GCCAGAAGTGGCTCTGAA	GATCTCCCGCAAGGCTGAA	cyclin-dependent kinase 9
CDKN1A	NM_078467.1	TGGAGACTCTCAGGGTCGAAA	CGGGCTTTGGAGTGGTAGAA	cyclin-dependent kinase inhibitor 1A (p21, Cip1)
CDKN1B	NM_004064.3	GCAATGCGCAGGAATAAGGAA	TTGGGGAACCTGCTGAAACA	cyclin-dependent kinase inhibitor 1B (p27, Kip1)
CFL1	NM_005507.2	GCTCAAGGACGCCATCA	TCCTTGACCTCTCGTAGCA	cofilin 1 (non-muscle)
CUL4A	NM_003589.2	TGCCGAAGGCCAAAGGTTAA	CTCCCTCTTCTTAAGCGTTTAC	culin 4A
DDI1	NM_001923.3	TTGGCTACAAGCCCATGGAA	CAAGATTTCCACAGCACTCATCC	damage-specific DNA binding protein 1, 127kDa
DDX58	NM_014314.3	GACTGGACGTGCCAAAACA	CTCCACTGGCTTGAATGCA	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58
DNAJB1	NM_006145.1	AAGGATCGTGTAGGCGCTA	TGGGGCCACTCCCTTTA	DnaJ (Hsp40) homolog, subfamily B, member 1
DNAJ1H1	NM_001376.4	GGAGTGTGCTGTGTTTAA	CAAGTGGCTTGAAGTGTGAC	dynein, cytoplasmic 1, heavy chain 1
E2F1	NM_005225.2	AGCTATTGCCAAGAGTCCAA	TCCTGGTCAACCCCTCAA	E2F transcription factor 1
EIF2AK2	NM_002759.2	TGCTAATTCAGGACCTCCA	TCACCTTCTGAAATCTCTCC	eukaryotic translation initiation factor 2-alpha kinase 2
ESRRA	NM_004451.3	GGAGCGAGGAGATGTTCTAC	CTTCTCGAGCTGCTCCA	estrogen-related receptor alpha
FAS	NM_000043.4	GCATCTGGACCTCTACC	CCTTGGAGTTGATGTCAGTAC	Fas cell surface death receptor
FOXO3	NM_001455.3	CGCTCTCTCCGCTGAA	TTTGAGGGCCACGTA	forkhead box O3
FOXP3	NM_014009.3	TGTGGGTAGCCATGGAAA	GGTGCATGTGTGGAA	forkhead box P3
GAPDH	NM_002046.4	GAACGGGAAGTGTGATCAA	ATCGCCCACTGATTTTGG	glyceraldehyde-3-phosphate dehydrogenase
HIF1A	NM_001530.3	CAGTGCACACGCCTGGATA	TTCTTGTGCTCATATCCATCAA	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
HK2	NM_000189.4	CTGCTGAAGGAAGCATCCA	TAGTCCGACTGTGCTGTAC	hexokinase 2
HMGAI1	NM_002131.3	CCGACCAAGGGAAGCAAAA	GTCTGCCCTTGTTTCC	high mobility group AT-hook 1
HSP90AA1	NM_001017963.2	ACTGCACAGAATGAAGGAAA	CCATGTTTCCGAGACGTTCC	heat shock protein 90kDa alpha (cytosolic), class A member 1
HSP90AB1	NM_007355.2	TCTCGCATGAAGGAGACACA	CGCACTGCTCCACAAA	heat shock protein 90kDa alpha (cytosolic), class B member 1
HSPA1A	NM_005345.5	AGGCTTCCAGAGCGAAC	GAGAAGAGCTGGTCTTCC	heat shock 70kDa protein 1A
HSPA1L	NM_005527.3	TGCATTAATCTGGACGTTTCCA	GGCGATCCCTGGCAGTA	heat shock 70kDa protein 1-like
HSPB1	NM_001540.3	AGCTGACGGTCAAGACAA	GTGTATTTCCGCGTGAAGCA	heat shock 27kDa protein 1
HSPH1	NM_006644.2	TGGAGTTGACGCAAAA	TGAATGCTGGCCATGAAA	heat shock 105kDa/110kDa protein 1
IFI16	NM_005531.2	GTGAATGGGTTGTTGAGTAC	CACCATTCACTTCCCTGTA	interferon, gamma-inducible protein 16
IFI6	NM_002038.3	TGCTACCTGCTCTTCA	TCAGGGCTTCCAGAAC	interferon, alpha-inducible protein 6
IFNA1	NM_024013.2	TGACTCATAACACAGGTAC	CAGGGGTGAGAGCTTTGAA	interferon, alpha 1
IFNB1	NM_002176.2	ATGAGCACTGTGCACCTGAA	GACTGTACTCTTGGCCTTCA	interferon, beta 1, fibroblast
IFNG	NM_000619.2	ACTGCCAGGACCATATGTA	GTTCATTATCCGTACATCTGAA	interferon, gamma
IL7R	NM_002185.2	GGAGAAAGTGGCTATGCTCAA	CTGCGTCCATCACTTCCA	interleukin 7 receptor
IRF1	NM_002198.2	AACAAGGATCGCTTGTTC	TGGGATCTGGCTTTC	interferon regulatory factor 1
IRF3	NM_001197123.1	ACCAATGGTGGAGGAGTAC	TGGGGCCAAACCATGTTA	interferon regulatory factor 3
IRF4	NM_002460.3	CACCATGACAACGCTTACC	CGAGGGTGGCATCATGTA	interferon regulatory factor 4
IRF7	NM_004031.2	GGCAGGACCGTACCTGTCA	ACCGTGGCCCTGTGA	interferon regulatory factor 7
KLF2	NM_016270.2	ATCCTGCCCTCTTCCA	CCATGGACAGGATGAAGTCC	Kruppel-like factor 2
MAPK1	NM_002745.4	TTGGTACAGGGCTCCAGAAA	TCTGCCAGAAATGAGCCTA	mitogen-activated protein kinase 1
MAPK3	NM_001040056.1	GCTACACGCGAGTGGAGTACA	GTCTTGGCAGTGGTCCATA	mitogen-activated protein kinase 3
MKI67	NM_001145966.1	AGAGTAACGCGGAGTGA	CTTGACACACATGCTCTCA	marker of proliferation Ki-67
MTOR	NM_004958.3	CCAAACCCAGGTGTGATCAA	TCCTCATTTCCAGGCCACTA	mechanistic target of rapamycin (serine/threonine kinase)
MX1	NM_001144925.1	ATGCTACTGTGGCCAGAAA	GGCCGACCTTCTCTCATA	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)
MX2	NM_002463.1	CGACTGGCAGAAAGACTTACC	CTTCTGGTGGCTTCCCTTA	myxovirus (influenza virus) resistance 2 (mouse)
MYC	NM_002467.4	CCTGGTGTCCATGAGGA	CCTGCCTTTTTCCAGAAA	v-myc avian myelocytomatosis viral oncogene homolog
MYD88	NM_001172567.1	CTCGAGGCAAGGAATGTGAC	TGTGGGAACTTCTTCTCA	myeloid differentiation primary response 88
NFATC1	NM_172390.1	TCCTTCCAACCAAAAGTCC	AGGATTCGGCAGAGTCAA	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
NFATC2	NM_012340.3	TGGAAGCCACGGTGGATA	TGTGCGGATATGCTTTC	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2
NFKB1	NM_001165412.1	CTACCTGGTGGCTTGTGAAA	ACCTTTGCTGGTCCACATA	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
OAS1	NM_016816.2	TACCCTGTGTGTGCTCAA	AGAGGACTGAGGAGACAAAC	2'-5'-oligoadenylate synthetase 1, 40/46kDa
PDE4A	NM_001111307.1	ATCACCCTGGGCTTCCAA	GAGCACCAGCTATCGTTGTA	phosphodiesterase 4A, cAMP-specific
PIK3CB	NM_006219.1	CGCTTCTGAACTGGCTTAA	CCAGCACAGGACAGTGTAAA	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit beta
PML	NM_033238.2	CCAACAACATCTTCTGCTCA	TTGGAACATCTCCGGCAGTA	promyelocytic leukemia
PPIA	NM_021130.3	TGCTGCCGTTTTGACAG	ACGGCAATGCGAAGAACAC	peptidylprolyl isomerase A (cyclophilin A)
PRDM1	NM_001198.3	CCTGGTACACACGGGAGAAA	TTGAGATTGCTGGTGTGTA	PR domain containing 1, with ZNF domain
PRKAA1	NM_206907.3	CCAACATGTGACACAGAA	AGAATAACCCCACTGCTCA	protein kinase, AMP-activated, alpha 1 catalytic subunit
PRKCA	NM_002737.2	ACCATTCCGCTCACACTAAA	AGTCTCGGTCTTGTGTA	protein kinase C, alpha
PSIP1	NM_021144.3	GGTTTATGGGAGATAGATAACAATCCA	CATGGTGGTATCTTCTTTGAA	PC4 and SFRS1 interacting protein 1
RAB7A	NM_004637.5	GTGGATGACAGGCTAGTACA	ACCTCTGAGAAGGCCACAC	RAB7A, member RAS oncogene family
RB1	NM_000321.2	TGCGCTTTGAGGTTGTA	TCAGAATCCATGGGAAGACAA	retinoblastoma 1
RHOA	NM_001664.2	GTGCCACAGTGTGGAGAA	TGTGTCCACAAGGCCAAC	ras homolog family member A
RICTOR	NM_152756.3	CTTCGGTGTGGAGGTTTATA	ACACAGCCTGCTTCTTCA	RPTOR independent companion of MTOR, complex 2
RPS6KB1	NM_003161.3	AGACCTGAAGCCGGAGATA	AGTCCAAAGTCTGTTAGTTTCA	ribosomal protein S6 kinase, 70kDa, polypeptide 1
RPTOR	NM_020761.2	GCTCAGAGCTGGAGGATGAA	AGGGTCCACCAACATTCA	regulatory associated protein of MTOR, complex 1
RRM2	NM_001165931.1	GCAGCAAGCGATGGCATA	GAAACAGCGGGTCTGTGAA	ribonucleotide reductase M2
SLC1A5	NM_001145145.1	CTCTACCACCTATGAAGAGAGAAA	CAGGATGTTATCCCTTCCA	solute carrier family 1 (neutral amino acid transporter), member 5
SLC2A1	NM_006516.2	ATTGTGGGATGTGCTTCC	AGAACCAGGAGCACAGTGA	solute carrier family 2 (facilitated glucose transporter), member 1
SLFN11	NM_152270.3	TCCAAGAAAGCCGTTGAA	TCTGAGATCAGTCCCTCCA	schlafen family member 11
SMARCB1	NM_003073.3	TCCACAACATCAACAGGAAC	AGCTGGGTATGGTTCATCAA	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1
STAT1	NM_007315.3	ATGCTGGCACAGAACGAA	GCTGGCACAAATGGGTTCAA	signal transducer and activator of transcription 1, 91kDa
STAT3	NM_003150.3	GGAAATAGTGGTGAAGTGTGAA	CCGAGTCAACTCCATGTCAA	signal transducer and activator of transcription 3 (acute-phase response factor)
STAT5B	NM_012448.3	AACAGAGTGTGGTCCGAGAA	CTTCTGGGACATGGCATCA	signal transducer and activator of transcription 5B
TLR3	NM_003265.2	TCTCATGTCCAATCAATCCA	CAGCTGAACCTGAGTCTCA	toll-like receptor 3
TLR7	NM_016562.3	TCTTCAACAGACCTCACTTCC	AGCCCCAAGAGTGGGAAA	toll-like receptor 7
TLR8	NM_138636.4	CTGCTGCAAGTTACGGAAATGAA	GCGCATAACTCACAGGAACC	toll-like receptor 8
TLR9	NM_017442.3	TGCAACTGGCTGTCTGAA	ACAAGGAAAGGCTGGTGA	toll-like receptor 9
TP53	NM_000546.5	GACTGTACCACCTCACTACA	AAAGCTGTTCCGTCAGTA	tumor protein p53
TRAF6	NM_004620.2	TGGCAATGTCTACTGTGAATAC	CTTATGGGACAGTCTAGATCA	TNF receptor-associated factor 6, E3 ubiquitin protein ligase
UNG	NM_080911.2	TGGACCCAGATGTGACATA	AGAGCCCGTAGCTGATTA	uracil-DNA glycosylase
VPRBP	NM_014703.2	TGGATGGCGGATGCTTGTATA	TTTATTGGCTTCCCGGAA	Vpr (HIV-1) binding protein
APOBEC3F	NM_145298.5	AGCCTGGAGCAGAAAGTAAA	ATCCTTGGCCGGCTAGTCC	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3F
APOBEC3G	NM_021822.3	CGCAGCCTGTGTGAGAAA	TTGCTCCAACAGCTGTGAAA	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G
GUSB	NM_000181.3	CATCGATGACATCACCTCAC	ACAGTTACTGCTTGTGACA	glucuronidase, beta
KLF4	NM_004235.4	CTGCGGCAAAAACCTACAAA	CGTCCAGTACAGTGGTAA	Kruppel-like factor 4 (gut)
SAMHD1	NM_015474.3	CCCGTGTCTGTAAGTAGACAA	TGGAGGTGGGAAATGTGTA	SAM domain and HD domain 1
SLC2A3	NM_006931.2	CCGTGGACTTCTGCTCAA	TAAAGCAGCCACAGTGA	solute carrier family 2 (facilitated glucose transporter), member 3
TREX1	NM_033629.3	ACCATCTGCTGTCAACCA	TCTCTCAAGGCTGGGACTA	three prime repair exonuclease 1
TRIM5	NM_033092.2	AGAAGCAGCAGGAAGCTGAA	GCCAAGACGTTGTTTGTCA	tripartite motif containing 5

Table S1

Code	Gender	Age	Viral load (RNA copies/ml plasma)	Months with undetectable viral load on ART	CD4 T cell count (cells/ $\mu$ l blood)	Cell- associated HIV DNA (copies/ $10^6$ CD4)
<b>034001</b>	M	57	<40	77	858	3805
<b>034004</b>	F	66	<40	110	593	3605
<b>034005</b>	M	50	<40	39	971	2797
<b>034006</b>	M	61	<40	77	750	8715
<b>034008</b>	M	33	<40	36	1119	4891
<b>L34001</b>	M	55	<40	195	693	NA

M, male; F, female; NA, not available.

Gene name	Stability value
BECN1	0,250549233
PRKAA1	0,288508569
RAB7A	0,30946967
TRAF6	0,319334695
TP53	0,328343002
VPRBP	0,365991082
API5	0,367327819
STAT3	0,370608212
EIF2AK2	0,384103082
HIF1A	0,391907917
TREX1	0,39253953
AKT1	0,394023857
RICTOR	0,395377783
MAPK1	0,396649613
PSIP1	0,408881136
CDKN1B	0,413957015
RPS6KB1	0,416878613
CCNT1	0,417659018
CDK9	0,426303822
MTOR	0,434251173
HMGAI1	0,436534261
CUL4A	0,454108452
MYC	0,457005393
MYD88	0,461562789
NFATC1	0,463277429
SAMHD1	0,467104121
CASP8	0,467443456
NFKB1	0,472349996
NFATC2	0,476133255
PRKCA	0,47821775
DDB1	0,482170786
PIK3CB	0,487302561
DDX58	0,496549071
BCL11B	0,498962754
PPIA	0,499844178
BCL2	0,502606509
MAPK3	0,51179285
HSPB1	0,511905741
RHOA	0,515493522
STAT5B	0,516400376
CASP3	0,536630196
HSP90AB1	0,538067061
IRF3	0,546799681
SMARCB1	0,552440933
APOBEC3F	0,55660686
IFI16	0,570759333
IRF1	0,572196628
HSPH1	0,587874332
MX2	0,590279919
SLC2A3	0,601232687
IFI6	0,602522338
IRF4	0,610056472
MX1	0,613977716
CFL1	0,620028452
IL7R	0,64055187
HSP90AA1	0,662468904
HSPA1A	0,665415349
SLFN11	0,6889147876
RB1	0,697664041
STAT1	0,702260041
FAS	0,703247399
DNAJB1	0,708604559
TRIM5	0,725969121
IRF7	0,734412588
FOXP3	0,739563525
SLC2A1	0,746229633
RPTOR	0,747416766
ACTB	0,759726907
GAPDH	0,768315184
DYNC1H1	0,77543431
APOBEC3G	0,776517129
HK2	0,794980726
GUSB	0,821595116
PRDM1	0,844574596
BCL2L1	0,867143178
UNG	0,89218868
CDKN1A	0,909445507
FOXO3	0,936183284
IFNB1	0,945277953
E2F1	0,993140533
RRM2	0,993363254
SLC1A5	1,128540312
ESRRA	1,228239862
MKI67	1,236841231
TLR9	1,23828716
IFNG	1,417604718
OAS1	1,614650004
KLF2	1,622314129
PDE4A	1,772367033
TLR8	1,903574476
IFNA1	2,132211213
TLR3	2,263468774
PML	2,39732875
TLR7	2,584686641
KLF4	2,652965521
HSPA1L	3,063617743

Better stability Value  
(Best House Keeping Gene)

Worse stability Value  
(Worse House Keeping Gene)

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