Universitat Autònoma de Barcelona

Characterization of cellular factors involved in HIV-1 pathogenesis with potential therapeutic implications in viral infections and cancer

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Certifiquen:

Que el treball experimental i la redacció de la memòria de la Tesi Doctoral titulada "Characterization of cellular factors involved in HIV-1 pathogenesis with potential therapeutic implications in viral infections and cancer" han estat realitzades per Marc Castellví Nadal sota la seva direcció i considera que és apta per a ser presentada per optar al grau de Doctor en Immunologia Avançada per la Universitat Autònoma de Barcelona.

I per tal que en quedi constància, signen aquest document a Badalona,

08 de setembre de 2021.

Dr. Bonaventura Clotet Sala

Dra. Ester Ballana Guix

A la meva família i a la Marta,

pel suport incondicional que m'han donat sempre

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SUMMARY

The main role of the human immune system is to eliminate cells presenting foreign antigens and abnormal patterns. However, this system can fail in eliminating the anomalies, leading to the establishment of chronic pathologies. Prototypical examples of immune system defeat are cancer and HIV-1 infection. In both conditions, the immune system and current pharmacological therapy eventually fails to eradicate the pathogenic anomaly, mainly due to the generation of latent viral reservoirs in HIV-1 infection, and to drug resistance in cancerous processes. Hence, this thesis evaluates the role of two key cellular factors, the Fc-gamma receptor CD32 and the triphosphohydrolase SAMHD1, to explore its function in disease progression and its potential in the development of novel therapeutic approaches in HIV/AIDS and cancer.

Through the study of *in vitro* models of acute and latent HIV-1 infection and in HIV-1+ patients, we have demonstrated that CD32, proposed as a marker of the latent HIV reservoir, is a marker of T cell activation either induced by exogenous stimuli or HIV-1 infection. Moreover, CD32 expressing cells are not preferentially infected despite of its activation state, and HIV-1 virions produced after stimulation of infected cells are equally infectious regardless of CD32 expression. Thus, CD32 does not represent a key marker of HIV-1 latent reservoir.

SAMHD1 is a viral restriction factor that controls intracellular dNTP pool and plays a key role in the first steps of HIV infection and latency establishment, but also influences efficacy of certain drugs used as anti-HIV or anti-cancer treatments. Here, we show that SAMHD1 is able to either enhance or limit the antiviral and anticancer activity of several antimetabolites currently used to treat cancer. Furthermore, we highlight the potential of the pharmacological modulation of SAMHD1 activity, which open the door to the development of novel therapeutic strategies in the treatment of cancer, viral infections, and immune diseases.

In summary, this thesis demonstrates that translational research directed towards *in vitro* study of cellular factors is a powerful tool for the validation of promising therapeutic targets.

RESUM

El paper principal del sistema immunitari humà es eliminar cèl·lules que presenten antígens forans i patrons anormals. No obstant, aquest sistema pot fallar a eliminar les anomalies, conduint a l'establiment de patologies cròniques. Exemples prototípics de la derrota del sistema immunitari son en càncer i la infecció per VIH. En totes dues condicions, el sistema immunitari i la teràpia farmacològica actual no aconsegueixen erradicar el patogen, principalment degut a la generació de reservoris virals latents en el cas del VIH i a la resistència a fàrmacs en el cas dels processos cancerígens. Així doncs, aquesta tesi pretén avaluar el paper de dos factor cel·lulars clau, el receptor Fc-gamma CD32 i la trifosfohidrolasa SAMHD1, per explorar la seva funció en la progressió de les malalties i el seu potencial en el desenvolupament de noves aproximacions terapèutiques en VIH/SIDA i càncer.

Mitjançant l'estudi *in vitro* de models d'infecció aguda i latent del VIH-1 i en pacients VIH-1+, hem demostrat que CD32, proposat com a marcador de latència del VIH, és un marcador d'activació de cèl·lules T induït per estímuls exògens o bé per infecció per VIH. A més, les cèl·lules que expressen CD32 no s'infecten preferentment tot i estar activades i el virions produïts per aquestes cèl·lules són igualment infecciosos independentment de l'expressió de CD32. Concloent que CD32 no marca cèl·lules T CD4+latentment infectades del reservori del VIH-1.

SAMHD1 és un factor de restricció viral que controla el pool de dNTPs intracel·lular i juga un paper clau en les primeres fases de la infecció del VIH i en l'establiment de la latència, però també influencia l'eficiència de certs fàrmacs utilitzats en el tractament del VIH i el càncer. Hem demostrat que SAMHD1 pot potenciar o limitar l'activitat antiviral i anticancerígena de diversos antimetabòlits utilitzats per tractar el càncer. A més, remarquem el potencial de la modulació farmacològica de l'activitat de SAMHD1, el qual obre la porta al desenvolupament de noves estratègies terapèutiques en el tractament del càncer, les infeccions virals i les malalties immunològiques.

En resum, aquesta tesi demostra que la traducció de la investigació dirigida cap a l'estudi *in vitro* de factors cel·lulars és una eina poderosa per a la validació de noves dianes terapèutiques.

RESUMEN

El papel principal del sistema inmunitario humano es eliminar células que presentan antígenos foráneos y patrones anormales. No obstante, este sistema puede fallar en eliminar las anomalías, conduciendo al establecimiento de patologías crónicas. Ejemplos prototípicos de la derrota del sistema inmunitario son el cáncer y la infección por VIH. En ambas condiciones, el sistema inmunitario y la terapia farmacológica actual no consiguen erradicar el patógeno, principalmente debido a la generación de reservorios virales latentes en el caso del VIH y a la resistencia a fármacos en el caso de los procesos cancerígenos. Así pues, esta tesis pretende evaluar el papel de dos factores celulares clave, el receptor Fc-gamma CD32 y la trifosfohidrolasa SAMHD1, para explorar su función en la progresión de enfermedades y su potencial en el desarrollo de nuevas aproximaciones terapéuticas en VIH /SIDA y cáncer.

Mediante el estudio *in vitro* de modelos de infección aguda y latente del VIH-1 y en pacientes VIH-1 +, hemos demostrado que CD32, propuesto como marcador de latencia del VIH, es un marcador de activación de células T inducida por estímulos exógenos o por la infección por VIH. Además, las células que expresan CD32 no se infectan preferentemente a pesar de estar activadas y los viriones que emergen de estas células son igualmente infecciosos independientemente de la expresión de CD32. Concluyendo que CD32 no marca células T CD4 + latentemente infectadas del reservorio del VIH-1.

SAMHD1 es un factor de estricción viral que controla el pool de dNTPs intracelular y juega un papel clave en los primeros pasos de la infección del VIH y el establecimiento de la latencia, pero además influencia la eficacia de ciertos fármacos utilizados en el tratamiento del VIH y el cáncer. Hemos demostrado que SAMHD1 puede potenciar o limitar la actividad antiviral y anticancerígena de diversos antimetabolitos usados en el tratamiento del cáncer. Además, remarcamos el potencial de la modulación farmacológica de la actividad de SAMHD1, que abre la puerta al desarrollo de nuevas estrategias terapéuticas en el tratamiento del cáncer, las infecciones virales y las enfermedades inmunológicas.

En resumen, esta tesis demuestra que la traducción de la investigación dirigida hacia el estudio *in vitro* de factores celulares es una herramienta poderosa para la validación de nuevas dianas terapéuticas.

ABBREVIATIONS

ADAR1	Double-stranded RNA-specific adenosine deaminase
ADCC	Antibody-dependent cell-mediated cytotoxicity
AGS	Aicardi-Goutières syndrome
AIDS	Acquired immunodeficiency syndrome
ALL	Acute lymphoblastic leukaemia
AML	Acute myelogenous leukaemia
APOBEC3	Apolipoprotein-B mRNA-editing catalytic polypeptide-like-3
ART	Antiretroviral therapy
AZT	Zidovudine
CA	Capsid
CCR5	C-C chemokine receptor 5
CD	Cluster of Differentiation
CDC	Center for Disease Control and Prevention
CDK	Cyclin-dependent kinase
CI	Combination index
COSMIC	Catalogue of Somatic Mutations in Cancer
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCR4	C-X-C chemokine receptor 4
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EC50	50% effective concentration
EFV	Efavirenz
FACS	Fluorescence-activated cell sorting
FCGR2A	Fc-gamma receptor FcγR-IIa
FDA	Food and Drug Administration
FU	Fluorouracil
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony stimulatory factor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HTLV	Human T-lymphotropic virus

IC	Inhibitory concentration
IFIH1/MDA5	Melanoma differentiation-associated protein 5
IFITM	Interferon-induced transmembrane gene family
IL	Interleukin
IN	Integrase
INF	Interferon
INSTI	Integrase strand transfer inhibitor
ISG	Interferon-stimulated genes
LAG-3	Lymphocyte activation gene 3
LAV	Lymphadenopathy-Associated Virus
LEDGF	Lens epithelium-derived growth factor
LOD	Limit of detection
LRA	Latency Reversing Agent
LTR	Long terminal repeat
MA	Matrix
MARCH8	Membrane Associated Ring Ch8
MDM	Monocyte-derived macrophages
NC	Nucleocapsid
Nef	Negative regulatory factor
NF-κB	Nuclear factor k-light-chain-enhancer of activated B cells
NK	Natural killer
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
NVP	Nevirapine
ORF	Open reading frame
PAI	Post-attachment inhibitor
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PD	Palbociclib
PD-1	Programmed cell death-1
PI	Protease inhibitor
PIC	Pre-integration complex

PLWH	People living with HIV
PMA	Phorbol 12-myristate 13-acetate
PR	Protease
PTX	Pemetrexed
Rev	Regulator of expression of virion proteins
RNA	Ribonucleic acid
RNASEH	Ribonuclease H
RT	Reverse transcriptase
SAMHD1	Sterile α motif and Histidine-aspartic acid domain-containing protein 1
SERINC	Serin incorporator 3/5
SIV	Simian immunodeficiency virus
SLFN11	Schlafen 11
SU	Surface
Tat	Trans-activator of transcription
TIGIT	T cell immunoglobulin and ITIM domain
TM	Transmembrane
TREX1	Three prime repair exonuclease 1
TRIM	Tripartite motif-containing protein
UNAIDS	Joint United Nations Programme on HIV and AIDS
Vif	Viral infectivity factor
VLP	Viral-like particles
Vpr	Viral protein R
Vpu	Viral protein U
Vpx	Viral protein X
VSV-G	Vesicular stomatitis virus G protein

INTRODUCTION

1. History and discovery of the Human Immunodeficiency Virus

Acquired Immune Deficiency Syndrome (AIDS) was first identified in June 1981 by the North American *Center for Disease Control and Prevention* (CDC) in Los Angeles, California [1]. Two years later in 1983, Luc Montagnier and Françoise Barré-Sinoussi at Pasteur Institute, isolated for the first time the etiological agent of several immune-related syndromes including AIDS [2]. This new agent belonging to the family of T-lymphotropic retrovirus, which they termed LAV (*Lymphadenopathy-Associated Virus*) or HTLV-III [2], was later confirm by others as the causative agent of AIDS [3, 4]. In 1986, the International Committee on the Taxonomy of Viruses renamed the novel LAV or HTLV-III as human immunodeficiency virus (HIV) [5]. According to the 2020 UNAIDS data report, the number of estimated people living with HIV is 38 million, 1.7 million newly infected cases and 690,000 AIDS-related deaths that year [6], being AIDS one of the most pressing health challenge of our time.

2. HIV-1 structure and composition

HIV comprises two species of lentiviruses, HIV-1 and HIV-2, that belong to the family of *Retroviridae*, being HIV-1 the more clinically relevant. The HIV-1 genome is encoded by a single-stranded, positive-sense, RNA molecule of approximately 9,8 kilobases with nine different open reading frames (ORF) that produce fifteen distinct proteins and is flanked by two identical 634 bp sequences named long terminal repeats (LTRs). Three of these ORF encode the Gag, Pol, and Env polyproteins, which are subsequently proteolyzed into individual proteins. The four gag proteins, MA (matrix), CA (capsid), NC (nucleocapsid), and p6, together with the two Env proteins, SU (surface or gp120) and TM (transmembrane or gp41), are structural components that make up the core of the virion and the outer envelope. The three Pol proteins, PR (protease), RT (reverse transcriptase), and IN (integrase), provide essential enzymatic functions and are also encapsulated within the viral particle. HIV-1 encodes six additional proteins, called accessory proteins, that can be found in the viral particle. These are, Vif, Vpr and Nef, which are not required for viral replication, but they help improving replication

efficiency; Tat and Rev, that provide essential gene regulatory functions; and Vpu, which indirectly assists in the virion assembly [7] (**Figure 1**).

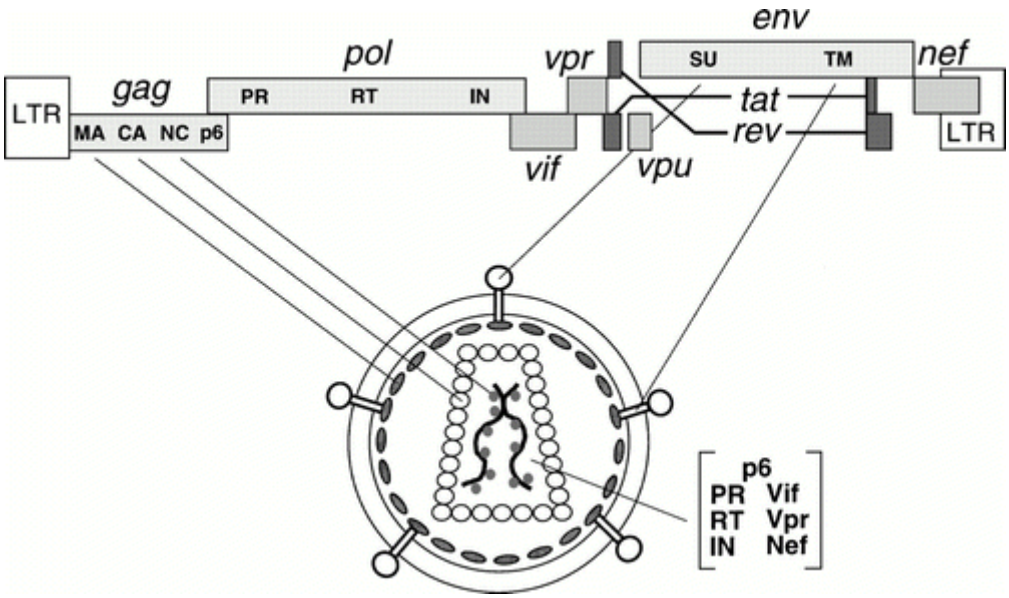


Figure 1. *Organization of HIV-1 genome and virion structure* [10].

Similar to HIV-1, HIV-2 also encodes Vif, Vpr, and Nef but lacks Vpu. Instead, HIV-2 and most SIVs encode a *vpx* gene, albeit Vpu and Vpx are not functional homologues and they target different host factors. While each accessory protein targets different cellular factors, the strategies employed are strikingly similar: none of the HIV accessory proteins has enzymatic activity; instead, they all seem to act as molecular adapters to manipulate the host cell, commonly resulting in the proteolytic degradation of the cellular target [8].

The tropism of HIV-1 is determined at the viral entry step into the cells when viral particles interact with CD4 as the primary receptor and then, with one of two different chemokine co-receptors, CCR5 or CXCR4, defining two distinct viral strains (R5 or X4). Thus, based on the differential expression of these co-receptors, HIV strains can preferentially infect naïve and memory T CD4+ lymphocytes, and to a lesser extent, macrophages, and dendritic cells (DCs) [9, 10].

3. HIV replication cycle

The replication cycle of HIV-1 begins when the viral particles enter target cells. The first entry step requires the binding of Env protein gp120 to CD4 receptor. This interaction induces the rearrangement of the variable loops of gp120 enabling binding to either chemokine CCR5 or CXCR4 co-receptors [11, 12]. Upon interaction of gp120 to CD4 and the corresponding co-receptor, the gp41 portion of Env inserts into the host membrane causing a fusion pore of the viral envelope with the plasma membrane of the target cell [13, 14].

Once fusion has occurred, the capsid of the virion is uncoated. Although it has been long considered that uncoating occurs in the cytoplasm after fusion-dependent entry and before nuclear import, the precise moment and location for this event it is still not clear. It has also been proposed that capsid remains intact post-entry, at least for the initiation of reverse transcription, and that uncoating occurs gradually during transport towards the nucleus [15, 16]. The viral RNA genome is reverse transcribed into double-stranded complementary DNA (cDNA) by viral RT, that is transported within the virus particle, and then imported into the nucleus through formation of HIV pre-integration complex (PIC) [17]. The PIC can cross the intact nuclear membranes of non-dividing cells through the nuclear pore complex without disrupting the nuclear envelope as a result of the function of Vpr [17-19]. In the nucleus, cDNA is integrated into the host genome by the viral integrase, and hereinafter, viral genome is defined as proviral DNA (**Figure 2**).

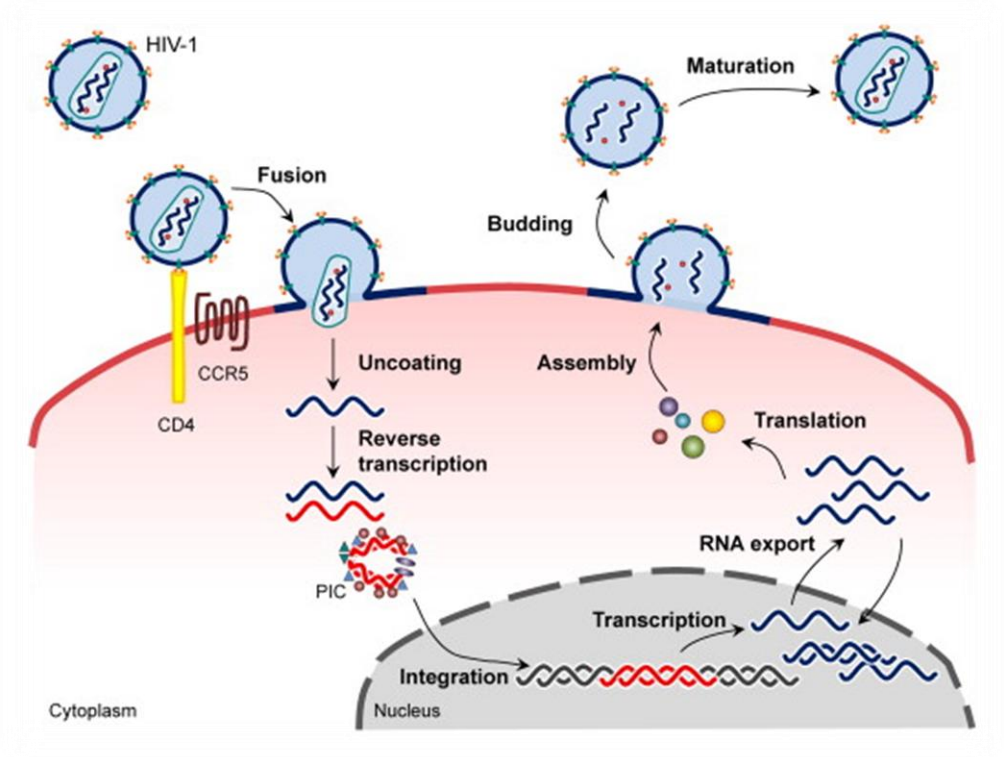


Figure 2. HIV-1 replication cycle. *The main steps of the replication cycle are shown binding to the CD4-receptor and co-receptors; fusion with the host-cell membrane; uncoating of the viral capsid; release of the viral genome and proteins; reverse transcription of the RNA into DNA; formation of the pre-integration complex (PIC); translocation into the nucleus. The viral DNA is integrated into the host DNA and is transcribed inside the nucleus. After export, RNA is translated to form new viral RNA and viral proteins that assemble at the host-cell membrane. New immature viruses bud from the host-cell and are released after which they mature, resulting in the production of new infectious virions [11].*

Proviral DNA is copied and transcribed using host machinery during cycles of cell division as part of cellular DNA, and viral RNA serves as a template for protein production as well as genomic RNA in progeny virions. Viral gene transcription is mainly controlled by Tat and Rev proteins, which act directly on viral RNA structures, enhancing viral transcription or allowing the translocation of singly spliced mRNAs from the nucleus into the cytoplasm, respectively [20-22].

Virion components need to traffic from their site of synthesis in the cytoplasm to the assembly site at the plasma membrane. This process and subsequent packaging of the virion components are driven by Gag and Gag-Pol polyproteins. Once assembled, the virion acquires its lipid envelope and Env protein spikes as it buds from the plasma membrane [17,23]. While budding of immature virion, viral maturation starts when protease is activated and cleaves Gag and Gag-Pol polyproteins into fully processed

proteins (MA, CA, NC, p6, PR, RT, IN). Once maturation is completed, viral particle is ready to infect a new host-cell and begin another replication cycle [23-25].

After integration, the provirus may become latent allowing the virus and its host cell to avoid detection by the immune system [26], a situation that will be discussed in detail in the following sections.

4. Natural history of untreated HIV infection

In the early days of the HIV epidemic, knowledge about the natural history of HIV accrued rapidly. However, the widespread use of effective antiretroviral therapy (ART) brought a shift in focus of the research community away from studies of natural history to those of treated infection [27]. HIV infection leads to progressive decline in CD4+ T-lymphocyte count increasing the risk for opportunistic infections and malignancies. Despite having a variable rate of progression determined by specific host and viral factors, the median time from infection to the development of AIDS ranges from 8 to 10 years among untreated individuals [28]. With the advent of ART, both morbidity and mortality have dramatically decreased. Overall survival and the rate of CD4-count recovery is influenced by age, baseline CD4 cell count, baseline viral load and initial and sustained viral suppression.

4.1. Acute HIV infection

Acute primary HIV infection is defined as the time period from initial infection with HIV to the development of an antibody response detectable by standard tests. Symptoms of acute primary HIV infection may be mild or severe and may last from a few days to several weeks, with the average duration being 14 days. The most common presenting symptom is fever, seen in over 75% of patients. Other commonly reported symptoms include fatigue, lymphadenopathy, headache, and rash [29, 30].

CD4 counts and CD4 function decline during primary HIV infection. While absolute CD4 count often rebounds after the primary infection it may not return to a normal baseline. In patients with clinical progression of HIV disease, CD4 responses against HIV itself appear to remain particularly impaired following primary infection [31].

4.2. Chronic HIV infection

After the period of acute HIV infection during which CD4 counts and viral load change dramatically, a relative equilibrium between viral replication and the host immune response is reached, and individuals may have little or no clinical manifestations of HIV infection. This time of clinical latency between initial infection and the development of AIDS may be long, averaging 10 years, even in the absence of treatment [32].

Despite the relative clinical latency of this stage of HIV infection, viral replication and CD4 cell turnover remain active, with millions of CD4 cells and billions of virions produced and destroyed each day [33]. During this period, most infected individuals will present a progressive loss of CD4 lymphocytes and subsequent perturbation of immune function [34-37]. On average, CD4 counts will drop by 50-90 cells/µL per year in asymptomatic individuals, usually with an acceleration of this rate over time [38].

4.3. Clinical AIDS

According to CDC criteria, AIDS is defined by either measurement of CD4 levels <200 cells/µL or by diagnosis of one of the AIDS-defining conditions, which include several opportunistic infections and different types of cancer. Progression to AIDS from time of infection occurs, on average, 2 years earlier when defined by laboratory criteria (CD4 levels <200 cells/µL) compared to clinical criteria (development of an opportunistic illness) [39, 40] (**Figure 3**). In the absence of treatment, the onset of the AIDS phase appears between 7 and 10 years or more from infection.

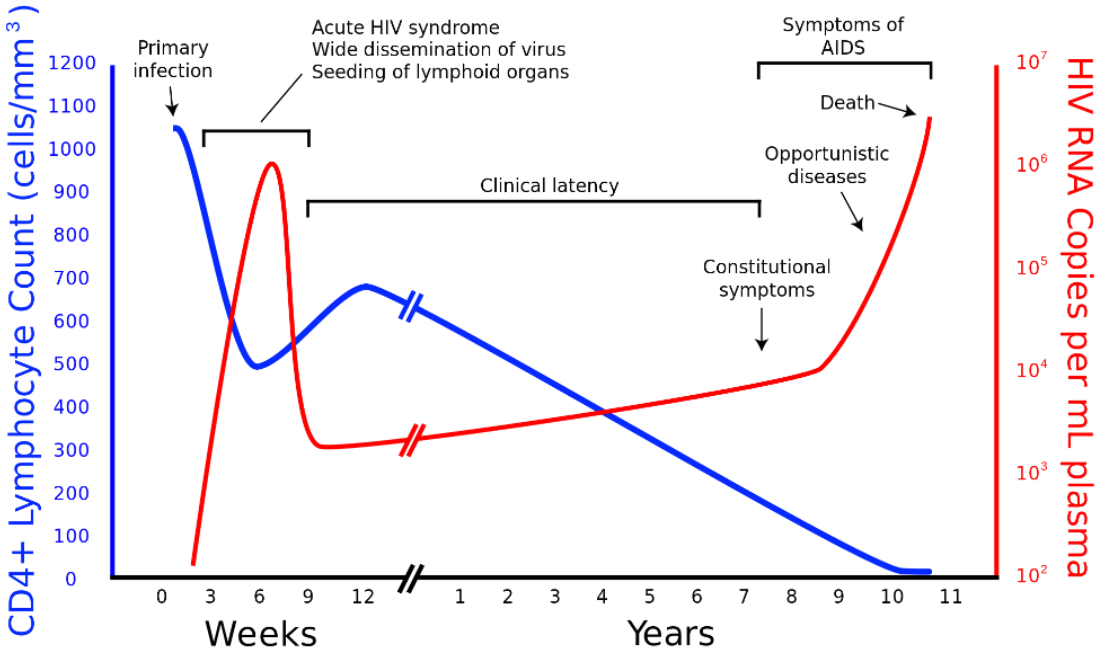


Figure 3. Natural history of untreated HIV. *In the absence of treatment, HIV-1 infection can be classified in 3 phases: Few weeks after primary infection, acute HIV syndrome occurs, characterized by a sudden increase on HIV-1 viremia and a decrease of the CD4+ T cell count, which leads to a wide dissemination of the virus and spread through lymphoid organs. Following, a chronic phase of clinical latency begins when HIV-1 viremia decreases due to the host immune system, and a partial recovery of the CD4+ T cells is observed, this phase can last for years and during this period CD4+ T count will progressively decrease in the absence of treatment. Finally, AIDS phase initiates when CD4+ T cells count is below 200 cells/µL, HIV-1 viremia raises leading to the apparition of constitutional symptoms and opportunistic diseases, which will ultimately lead to the death of the infected individua [41].*

4.4. Clinical AIDS under Antiretroviral therapy

Since the FDA approval of the first antiretroviral compound, AZT [42], in 1987, advances in ART have changed the perspective of HIV-1 infection from a lethal illness to a manageable chronic disease [43]. Nowadays, the use of combination therapy suppresses viral load below the limit of detection (LOD) (<50 copies of viral RNA/mL) following a four-phase decay of viremia, reaching stable level of viremia below LOD, that ideally should last for an unlimited period of time [44, 45] (**Figure 4**):

- Phase I: Initial dramatic decrease of viremia caused by the clearance of free virus (t½=6 h) and short-lived productively infected CD4+ T cells (t½=1–2 days).
- Phase II: The second phase of decay (t½=1–4 weeks) is less dramatic that in phase I, and is thought to reflect the loss of infected cells that are more resistant to

HIV-1 cytopathic effect or have longer half-life, such as partially activated T cells or macrophages.

- Phase III: Slower decrease of viremia due to the clearance of cells with even longer half-life. This phase has a half-life of approximately 273 days and viral RNA copies/mL are already below clinical LOD.
- Phase IV: Levels drop to a stable set point showing no evidence of further decay. Viremia persists at this stable set point for at least 7 years following the initiation of ART and reflects the remarkable stability of the long-lived cellular reservoirs that maintain residual viremia.

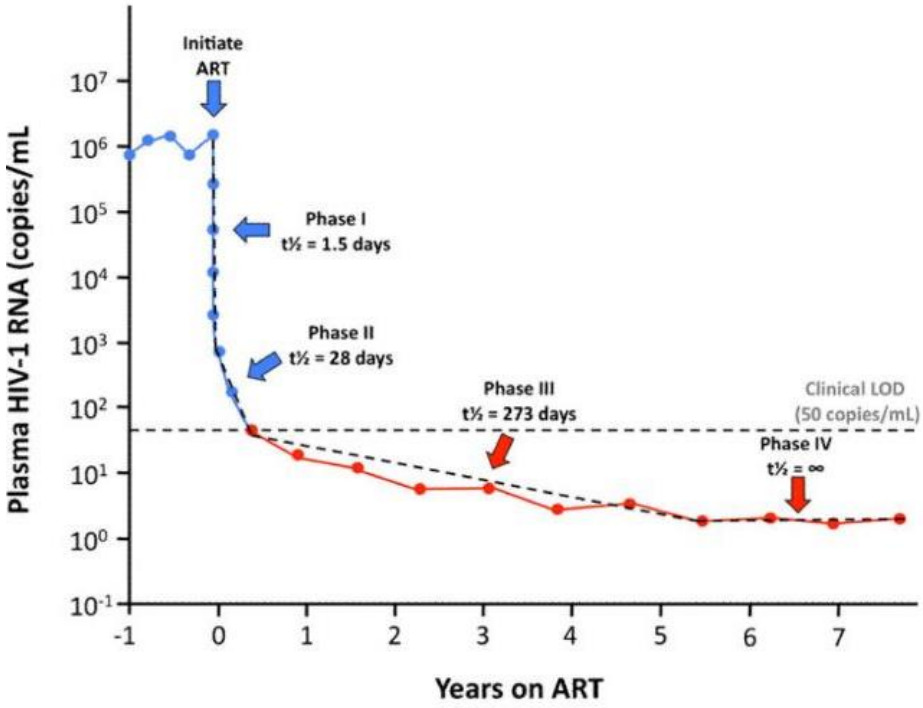


Figure 4. Decay dynamics of plasma HIV-1 RNA during ART. *Upon initiation of ART, viremia decays in multiple overlapping phases, which reflects the turnover of cells infected prior to ART with different half-lives [44].*

This decreased replication allows the treated individuals to control viremia, delay disease progression, prevent transmission and partially recover the CD4+ T cell count indefinitely [46]. However, upon treatment failure, virus replication increases again and CD4+ T cell count plummets, as observed in the HIV-1 acute phase. Treatment failure is caused by the acquired resistance to treatment due to the high mutagenesis rate of the RT, resulting in the appearance of resistant quasispecies in the presence of ART [47]. In

an effort to avoid this drug resistance, current combination therapy uses three antiretrovirals targeting at least two different steps of the viral cycle, thus, the chances of the virus to evolve and become resistant to the three drugs are reduced [48].

Based on their molecular targets, current HIV-1 antiretrovirals can be classified into seven different classes that include:

- Nucleoside reverse transcriptase inhibitors (NRTIs)
- Non-nucleoside reverse transcriptase inhibitors (NNRTIs)
- Integrase strand transfer inhibitors (INSTIs)
- Protease inhibitors (PIs)
- Fusion inhibitors
- CCR5 antagonists
- Post-attachment inhibitors (PAIs)

Combination therapy must be taken in combination and without interruption for a long-lasting effect. Although current ART is able to successfully control viral replication, it is unable to target those cells in which HIV-1 remains silent, the HIV-1 latently infected cells [49]. Due to this incomplete clearance of the infection, HIV-1 is able to rebound after ART discontinuation, independently of the time spent under treatment.

Long-term ART is also linked to a persistent immune activation and inflammation [50] as well as to toxicities associated with the treatment. Therefore, there is still an urgent need to develop novel therapeutic strategies to achieve an effective HIV-1 cure, defined as a treatment that should be able to induce a sustained remission of the virus after ART discontinuation.

5. HIV-1 latency and viral reservoir

Antiretroviral therapy suppresses HIV replication and improves immune function allowing the management of most HIV infected individuals. This represents the major success in AIDS prevention and a drastic reduction of the virus transmission risk. Unfortunately, ART treatment is not a cure to HIV since interruption of therapy inevitably leads to a rapid rebound of viral load to pre-ART levels. The cause of this therapeutic obstacle is the existence of latent viral reservoirs which harbour silent integrated viral HIV DNA capable of reactivate and produce viral particles. This complex and challenging viral hallmark is the main roadblock for achieving an HIV cure [27-30].

Viral reservoirs are heterogeneous and dynamic in nature [51]. Commonly, HIV reservoirs are defined as cell types or anatomical sites where a replication-competent form of the virus persists for a longer time than in the main pool of actively replicating virus [49]. This definition mainly restricts the viral reservoir to latently infected resting CD4+ memory T cells carrying stably integrated, transcriptionally silent but replication-competent proviruses [52, 53]. While in resting state, these cells do not produce viral particles; however, they can give rise to infective viruses following re-activation by different stimuli, causing a viral rebound when ART is stopped [54-58] (**Figure 5**). Nonetheless, it has also been proposed a wider definition of HIV reservoir that include all infected cells and tissues containing all forms of HIV persistence that can participate in HIV pathogenesis [56]. This alternative definition arises from the evidence that some defective provirus, unable to reignite infection, may still elicit immune activation through viral protein or novel antigen production and thus, taking part in residual HIV pathogenesis [59, 60]. Therefore, the eradication of this fraction of the HIV reservoir must be considered in the context of achieving a permanent cure.

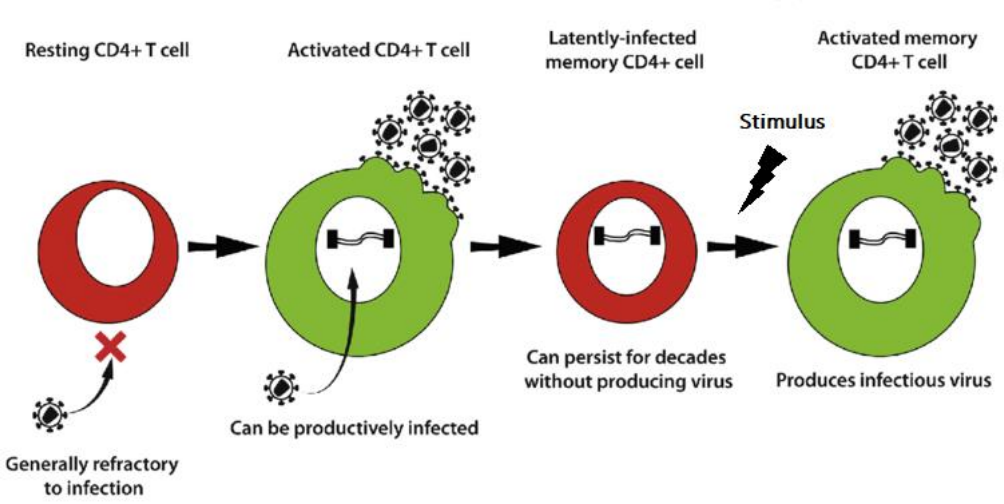


Figure 5. Model for the establishment of latent HIV-1 infection in resting memory CD4+ T *cells Resting CD4+ T cells cannot generally support HIV replication. However, if the cells are stimulated by cytokines or recognition of their cognate antigen then they become activated and susceptible to HIV infection. Infection will generally result in death of the host cell, but a small subset of these cells will transition back to a resting state before they can be killed by the virus. The result is a shut-down of HIV expression and production of a long-lived latently-infected cell that harbors an HIV provirus that is not producing viral proteins. Subsequent activation of this latently infected cell (perhaps many years later) results in re-initiation of virus expression and production of new infectious virions. [58].*

Several therapeutical strategies to eradicate latently infected cell has been proposed although with limited or no success in clinical trials. The “shock and kill” strategy is based on the uses of Latency Reversing Agents (LRAs) to increase HIV transcription, protein expression and virion production. In consequence, the latently infected cells may potentially die through virus-mediated cytopathic events or immune-mediated clearance [61-64].

5.1. Mechanisms of HIV-1 Latency

The mechanisms underlying the establishment and maintenance of HIV latency probably vary from one patient, one cell type, one tissue or one anatomical compartment to the other [65]. HIV predominantly targets CD4+ T lymphocytes, monocytes/macrophages, and dendritic cells, although additional cell types can also be infected and may contribute to the viral reservoir, such as natural killer (NK) cells and other specialized cell populations derived from various tissues, for example, renal, mucosal, and cervical

epithelial cells, gut-associated lymphoid tissues, skin fibroblast, bone marrow stem cells or mastocytes, astrocytes, and microglia in the central nervous system [66].

Multiple mechanisms acting in concert are involved in the establishment of HIV latency and operate mostly at the transcriptional level and at several post-transcriptional steps. HIV-1 latency results in a complex and variable combination of multiple elements acting at the initiation and/or at the elongation phase of transcription. After viral entry, HIV DNA is integrated into chromatin in a non-random process. The cellular lens epithelium-derived growth factor (LEDGF/p75) binds both cellular chromosomal DNA and HIV integrase and directs integration preferentially to introns of actively transcribed genes [67, 68]. Despite this, several mechanisms impede promoter activity, including steric hindrance, enhancer trapping and promoter occlusion depending on the orientation of the HIV-1 genome within the cellular transcriptional unit [56, 69]. This heterogeneous and dynamic combination of transcriptional repression mechanisms impedes the synthesis of the viral trans-activating factor Tat, a viral protein indispensable for activation of HIV-1 transcription [57].

In addition, several epigenetic modifications could contribute to the transcriptional silence and latency of HIV-1. In eukaryotic cells, DNA is wrapped around a nucleosome composed of a histone octamer. The histone tails are subject to multiple post-translational modifications including acetylation, phosphorylation, SUMOylation, ubiquitination and methylation. These reversible epigenetic marks regulate gene expression by altering chromatin condensation, allowing or blocking the accessibility of DNA to transcription factors and transcription machinery. The chromatin structure and the epigenetic control of the HIV-1 promoter (5'LTR) are key mechanisms underlying transcriptional regulation and thus latency [55]. Similarly, DNA methylation at cytosines located in CpG regions of promoter also participate in HIV-1 transcriptional silencing [70].

Altogether, this knowledge demonstrates the highly heterogeneous nature of HIV-1 reservoirs and highlight the urgent need to discover cellular markers of latently infected cells to be able to understand the complex and dynamic nature of viral reservoirs and to allow their selective targeting for eradication [71, 72].

5.2. Cellular Markers of HIV latency

So far, no reliable cellular marker has been discovered capable of identifying HIV reservoirs. Nonetheless, some molecules have been proposed and their further characterization could represent a significant step forward towards a better comprehension of the HIV reservoirs.

During chronic viral infections, T cells are constantly over-stimulated due to high antigenic production, leading to a progressive loss of function, and T-cell exhaustion [73-75]. High levels of different inhibitory receptors known as immune checkpoint molecules (ICs), are overexpressed during this period resulting in the suppression of immune response. Some of these ICs include programmed cell death-1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), lymphocyte activation gene 3 (LAG-3) and T cell immunoglobulin and ITIM domain (TIGIT), among others. Importantly, it has been shown that CD4+ T cells expressing these markers positively associate with the frequency of cells harbouring integrated HIV-1 DNA and are enriched for HIV-1 infection in several memory CD4+ T-cell subsets during ART [76, 77].

Similarly, using an *in vitro* primary CD4+ T-cell model for HIV post-integration latency, Iglesias-Ussel et al. identified CD2 as a putative marker for latently infected CD4+ T cells. They show that resting memory CD4+ $CD2^{high}$ T cells harboured higher HIV-1 DNA copies compared with the other cell subsets. Further, these cell population could be stimulated to express high levels of HIV-1 RNA, although no evidence of productive infection was provided [78, 79].

Furthermore, Hogan et al. demonstrated that CD30+ CD4+ T lymphocytes were significantly enriched for cell-associated HIV RNA but not HIV DNA in several individuals independently of ART use [80]. Moreover, they show the co-localization of HIV transcriptional activity and CD30 expression in gut-associated lymphoid tissue from both ART-treated and untreated individuals. Besides, using an anti-CD30 antibody conjugate on an *ex vivo* culture of peripheral blood cells, they observed a significant reduction in total HIV DNA, overall suggesting that CD30 could be a useful therapeutic target [81].

Serra-Peinado et al. reported a modest increment of HIV RNA positive cells among a small subpopulation of CD4+ T cells expressing the surface protein CD20. In addition, *ex*

vivo treatment of primary peripheral blood mononuclear cells (PBMCs) from ART-suppressed individuals with rituximab, an anti-CD20 monoclonal antibody, in combination with latency-reversing agents reduced the pool of HIV RNA+ cells [82].

At the beginning of this PhD thesis, Descours et al. identified 103 overexpressed genes in HIV+ resting cells in culture, 16 of which encode for transmembrane proteins and thus, might represent putative markers of HIV latently infected cells. The most highly transmembrane expressed gene was FCGR2A, which encodes the Fc-gamma receptor FcγR-IIa (CD32). The authors showed that CD32+ cells from HIV-1 positive participants were enriched in HIV DNA and inducible replication competent virus, concluding that CD32 is a cell surface marker of the CD4+ T cell HIV reservoir in HIV-infected virally suppressed participants. In contrast to all the markers previously suggested, in which HIV DNA enrichment was modest, Descours et al. showed a high enrichment in HIV DNA in CD4+ T cells with high CD32 expression as compared to CD32- CD4+ T cells [83].

CD32 represents a link between the humoral and cellular immune responses by triggering several functions, such as endocytosis and antibody-dependent cell-mediated cytotoxicity (ADCC) [84]. CD32 is a low-affinity Fc receptor with specificity for IgG antibodies and is commonly expressed on most myeloid cells, including monocytes, macrophages and eosinophils, but also in natural killer (NK) cells and B-lymphocytes [85-88]. CD32 is tightly regulated by agents such as phorbol 12-myristate 13-acetate (PMA) and cytokines, including interferon gamma (IFN-γ) and granulocyte-macrophage colony stimulatory factor (GM-CSF) [85-88]. The regulation of innate immune response recruitment is an important function of IgG-binding receptors such as CD32 [89]. Specifically, CD32 triggers phagocytosis and Antibody-dependent Cellular Cytotoxicity (ADCC), which explains its constitutive expression in macrophages and NK cells [56,57]. Notably, CD32 was shown to be significantly downregulated on the surface of multiple innate immune cell subsets in both treated and untreated HIV-1 infections. This downregulation could result in irreversibly reduced ADCC activity in progressive infection, even in the absence of active viral replication [90, 91, 92].

The role of CD32 in HIV latency is controversial and a matter of debate [92, 93, 96, 97]. If true, the finding that CD32 expression is a marker of a CD4+ T cell HIV-1 reservoir

would likely significantly impact the development of cure-focused HIV diagnostics and treatments [98] and thus it deserves careful consideration.

6. Restriction factors of HIV infection

The establishment and maintenance of HIV latency is a complex process involving a great variety of viral and cellular factors acting in concert. Hence, the characterization of the interplay between cell-intrinsic antiviral responses and HIV persistence could help to understand the role of host factors in HIV latency and may lead to novel approaches to reduce the size of the viral reservoir [99].

As mentioned above, HIV-1 requires the concerted contribution of diverse positively acting cellular factors and pathways to achieve efficient replication and maintenance, a trait shared with all viruses [99]. Conversely, mammalian cells also expressed numerous dominantly acting proteins directed to suppress viral replication. These have been termed restriction factors and they provide one of the first lines of defence against infection as a component of, or even preceding, innate antiviral responses [100]. Thus, restriction factors are host cellular proteins contributing to the frontline defence against viral infections. Restriction factors recognize and interfere with specific steps of the replication cycle of viruses, thereby blocking infection. They are generally interferon-inducible and their inherent features, such as constitutive expression in different cell types, self-sufficient activity, and rapidity of action, confer a potent and early restriction of viruses [101].

HIV-1 and HIV-2 have evolved distinct strategies to counteract the potent inhibitory activity of restriction factors in human cells, thereby allowing the virus to achieve efficient replication levels. The viral counteraction mechanisms from restriction factors are virus encoded and frequently, but not always, involve HIV regulatory/accessory proteins such as Vif, Nef, Vpu and Vpr; or Vpx for HIV-2. The need to escape from intracellular resistance appears to have been an important driving force behind the acquisition of these viral genes.

Recent years have witnessed a substantial increase into the research and discovery of new mechanisms of defence that act against viral infections and a great variety of innate host factors have been described (reviewed in 73). The thorough study of restriction factors generates new insights into the key molecular determinants of viral replication and reveal a roadmap of HIV-1 vulnerabilities that could lead to the development of new therapeutic targets. Host restriction factors can be classified based on their mechanism of action over the different phases of HIV life cycle (**Figure 6**):

- *Restriction factors acting on HIV entry step.*

Interferon-induced transmembrane gene family (IFITM). IFITM1 is mostly found on the plasma membrane and acts during the fusion phase of HIV-1 virions [102]. The *IFITM* genes are thought to inhibit HIV-1 entry by changing the composition and curvature of the plasma membrane, perhaps reducing its fluidity, thereby interfering with hemifusion [103, 104], a phenomenon essential for the incorporation of an HIV-1 virion into a target cell [105]. Although one of the requirements for considering a host protein a restriction factor is the presence of a counteracting protein in the HIV virions, it has been reported that HIV can develop resistance to some restriction factors in the absence of specialized accessory proteins. There is evidence that certain HIV-1 variants may be resistant to the restriction of IFITM proteins. In particular, transmitter/founder viruses have been found to be more resistant to IFITM restriction than viruses isolated from later times during infection; the later viruses may gain sensitivity as a result of escape from concomitant neutralizing antibody responses [106].

Serine incorporator 3/5 (SERINC). SERINC3 and 5 are cell surface proteins that also restrict HIV infection by modifying the lipid composition of the viral envelope, affecting membrane fusion step, specifically by incorporating serine into membrane lipids, most notably in sphingolipids and phosphatidylserine [107]. The accessory protein Nef is able to counteract SERINC3/5 restriction favouring HIV replication.

- *Restriction factors acting on capsid disassembly.*

Tripartite motif-containing (TRIM) proteins. TRIM are multi-domain proteins that act during the disassembly step of HIV entry phase. Some of which, including TRIM5α, also possess a C-terminal PRY/SPRY domain that is important for HIV-1 capsid recognition

[109]. It specifically binds the CA lattice of HIV-1 and induces premature disassembly of viral particles, accompanied by proteasomal-degradation of viral components such as integrase [110]. Nonetheless, because human TRIM5α has only modest activity against HIV-1, it does not drive viral adaptation. However, several reports have suggested that HIV-1 could acquire adaptive mutations, in the setting of an engineered TRIM5α molecule that did have the capacity to restrict HIV-1 replication [111, 112].

- *Restriction factors acting on HIV reverse transcription:*

Apolipoprotein B mRNA Editing Catalytic Polypeptide-like (APOBEC). APOBEC3 family members are cytidine deaminases induced by type I IFN that play important roles in the control of multiple retroviruses through RNA binding or through deamination of single-stranded DNA (ssDNA) [113, 114]. The APOBEC3 family members restrict HIV-1 by hypermutating its genome resulting in premature stop codons and defective proviruses that are incapable of propagating infection [115, 116]. The viral infectivity factor (Vif) is an accessory protein found in several lentiviruses including HIV and acts by disrupting the antiviral activity of the restriction factor APOBEC3 by targeting it for ubiquitination and proteasomal degradation [117-119].

Sterile alpha-motif (SAM) and Histidine-Aspartate (HD) Domain Containing Deoxynucleoside Triphosphate Triphosphohydrolase 1 (SAMHD1). SAMHD1 is a triphosphohydrolase with dNTPase activity that restrict HIV-1 by diminishing the intracellular pool of available dNTP in immune cells needed for HIV cDNA synthesis during reverse transcription [120-122]. SAMHD1 has been a topic of intense study in the present work and therefore, its characteristics and function will be discussed in detail in the following chapter.

- *Restriction factors acting on nuclear import, transcription, and translation of HIV genome.*

Mx proteins. MX2 is a member of a family of dynamin-like GTPase that appears to act at a late post-entry step prior to integration of proviral DNA, possibly through inhibition of nuclear import following reverse transcription, or by inhibiting the uncoating of HIV-

1 [126-128]. In addition, mutations in the HIV-1 capsid protein and in integrase confer resistance to MX2 [129].

Tripartite motif-containing (TRIM) proteins. TRIM22, a human paralog of TRIM5α, is involved in type I IFN-mediated restriction of HIV-1 replication [130, 131]. TRIM22 inhibits HIV-1 replication by interfering with Tat-and NF-κB-independent LTR-driven transcription, and by preventing Sp1 binding to the HIV-1 promoter [132, 133]. In addition to its role in blocking HIV-1 transcription, TRIM22 may also interfere with virion assembly and release by preventing the trafficking and budding of Gag proteins and Gag-containing virus particles [130].

Schalfen (SLFN). SLFN11 belongs to the Schlafen family and restricts HIV by binding to tRNAs, limiting their availability and thereby inhibiting the expression of viral proteins [134]. SLFN11 was also recently found to be significantly elevated in CD4+ T cells from elite HIV controllers as compared to non-controllers and ART-suppressed individuals, suggesting that SLFN11 may play a role in the suppression of HIV-1 *in vivo* [135].

Restriction factors acting on virion assembly and budding.

Tetherin. Tetherin, also known as BTS2, is a restriction factor localized in lipid rafts at the plasma membrane, in the trans-Golgi network, and in early recycling endosomes [136]. Tetherin inhibits the release of nascent HIV-1 particles by tethering the budding virions at the cell surface [137]. Nascent virions anchored to the membrane are then internalized and degraded in the lysosome. Vpu, a small transmembrane protein, interacts directly with tetherin at the trans-Golgi network and targets it for proteasomal or lysosomal degradation [138, 139]. Deletion of Vpu results in tetherin-mediated retention of virions at the plasma membrane [140]. In addition, Vpu also inhibits the activation of NF-κB by tetherin [141]

Membrane Associated Ring Ch8 (MARCH). MARCH8 blocks the incorporation of HIV-1 envelope glycoprotein into virus particles, resulting in a substantial reduction in the efficiency of virus entry, thus inhibiting its infectivity. Intriguingly, viruses are normally released, but are rendered non-infectious in the presence of MARCH8. Neither HIV-1 Vpr, Vpu nor Nef have detectable anti-MARCH8 activity, suggesting that HIV-1 lacks a counter-mechanism that dampens the effects of MARCH8. Studies are ongoing to

determine the *in vivo* relevance of MARCH8, and whether HIV-1 can indeed adapt resistance to its effects [108].

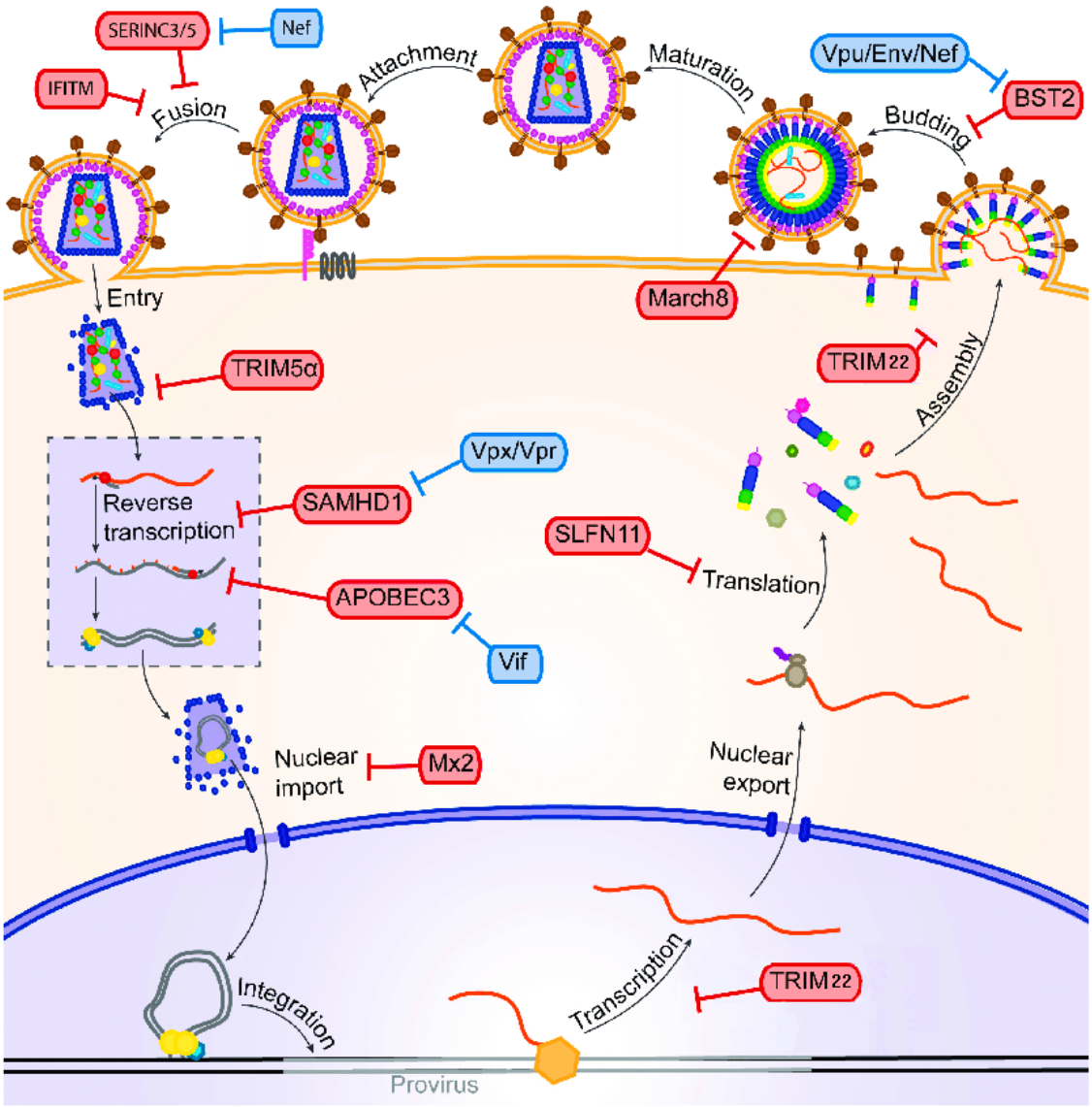


Figure 6. Schematic representation of host restriction factors and viral counterparts during HIV life cycle. *Cellular restriction factors (represented by red T bars) and the viral accessory proteins that counteract these factors (represented by blue T bars) are shown [Adapted from 142].*

7. SAMHD1 roles in health and disease beyond viral restriction

SAMHD1 was identified in 2011 as a restriction factor by two independent groups using a mass spectrometry pull-down approach to identify proteins that co-immunoprecipitated with the viral protein Vpx present in HIV-2, but not HIV-1 [123, 124]. SAMHD1 is a deoxynucleotide triphosphate (dNTP) hydrolase that catalyses the hydrolysis of canonical dNTPs into its constituent nucleoside and inorganic triphosphate [120]. Through its dNTPase activity, SAMHD1 maintains the intracellular dNTP pool at proper level for DNA replication and repair but below a potentially mutagenic threshold [150].

SAMHD1 inhibits retroviral replication at the reverse transcription (RT) step by maintaining the intracellular concentration of dNTP below the threshold required for reverse transcription of the viral RNA genome into DNA. Vpx from HIV-2 counteracts SAMHD1 function through the induction of SAMHD1 degradation by ubiquitination, leading to the increase of intracellular dNTP levels, and allowing the virus to retrotranscribe and replicate its genetic material [143, 144] (**Figure 7**).

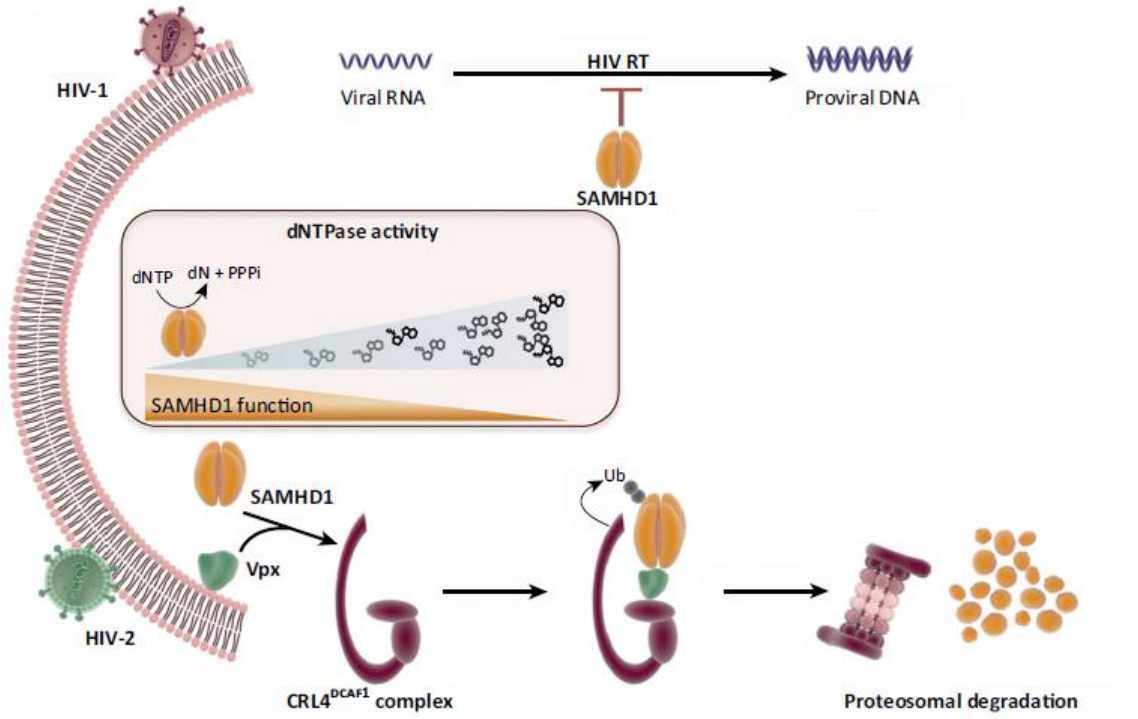


Figure 7. SAMHD1 dNTPase function and HIV restriction. *Proposed model for SAMHD1-dependent restriction of HIV-1. HIV-2 vpx promotes SAMHD1 degradation by recruiting it to the E3 ligase complex (CRL4DCAF1), inducing its ubiquitination and degradation by the proteasome [Adapted from 145].*

Whether HIV-1 has evolved adaptations to counter SAMHD1 is still not well understood, although Kyei et al. reported that HIV-1 might neutralize SAMHD1 in macrophages in concert with the cell cycle regulator cyclin L2 [125]. In addition to its role as a restriction factor of HIV-1 and 2, it has also been reported to restrict infection of Simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), bovine immunodeficiency virus (BIV), equine infectious anaemia virus (EIAV), murine leukaemia virus (MLV), Mason Pfizer monkey virus (M-PMV), Rous sarcoma virus (RSV), human T cell leukaemia virus type 1 (HTLV-1), vaccinia virus (VACV) and Herpes simplex virus type 1 (HSV-1) [146-149].

SAMHD1 is 626 amino acid protein comprise of an N-terminal sterile alpha motif (SAM) and a Histidine-aspartic acid containing Domain (HD). Although the role of the SAM domain remains unclear, this kind of domains are commonly involved in protein-protein and protein-DNA/RNA interactions [151]. A nuclear localization signal precedes the SAM domain and drives its nuclear localization [152, 153]. The HD domain is defined by the acid aspartic and histidine residues coordinated by a quartet of metal ions within the enzyme active site. Proteins containing HD domains are part of a superfamily of phosphohydrolases commonly involved in nucleic acid metabolism [154]. The HD domain of SAMHD1 contains the dNTPase active site, regulatory sites, and the necessary interfaces for enzyme oligomerization. The C-terminus of SAMHD1 is important for stabilizing the oligomeric state of the enzyme and nucleic acid interaction [155-158].
SAMHD1 exists in a monomer-dimer equilibrium and only tetramerizes when nucleotides bind to its regulatory sites and activate the catalytically competent holoenzyme [157, 159, 160]. Each SAMHD1 monomer contains two allosteric regulatory sites (RS1 and RS2) and activating nucleotide triphosphates must sequentially bind at each site to induce a conformational change that promote tetramerization and subsequent catalytic activation [159, 156, 157]. The RS1 pocket residues are structurally disposed to only allow the binding of (deoxy)guanosine triphosphate nucleotides [159, 161-163]. In contrast to RS1, RS2 presents a less restrictive binding site. RS2 can accommodate any of the four canonical dNTPs and occurs when intracellular dNTP concentrations are high enough to achieve the activating range [159, 164-166] (**Figure 8**). The binding event of a dNTP at RS2, which is preceded by docking of GTP in the

guanine specific RS1 pocket, stabilizes the tetrameric structure, thus the subunit assembly results in the formation of four regulatory clefts comprises of an RS1 and RS2 from adjacent monomers, as well as residues of a third SAMHD1 subunit [167]. Binding of activating nucleotides and the subsequent formation of the tetramer result in conformational changes that remodel the active site allowing substrate binding and catalysis.

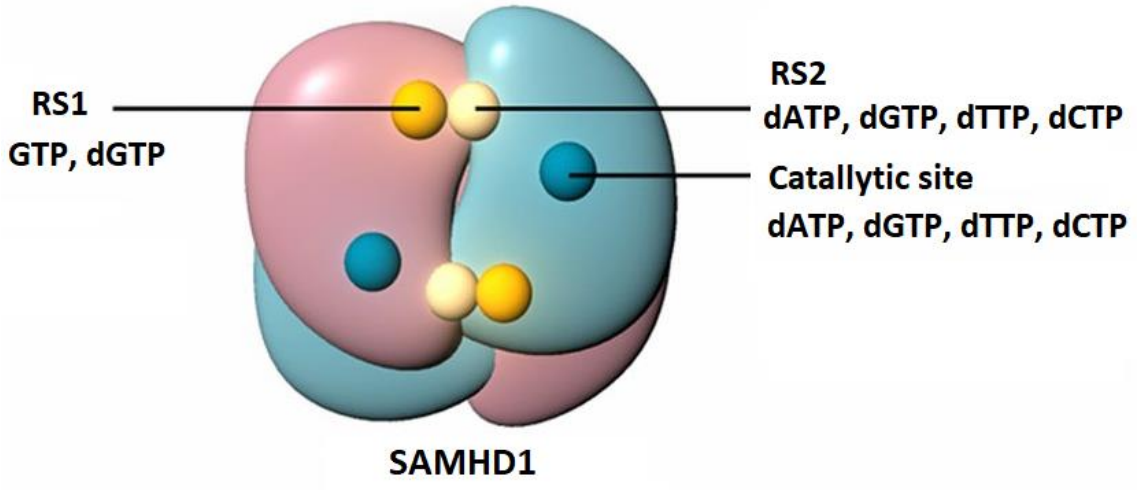


Figure 8. Schematics of SAMHD1 catalytic and regulatory sites. *Regulatory Site 1 (RS1) is able to accommodate only (deoxy)guanosine triphosphate nucleotides, while Regulatory Site 2 (RS2) and catalytic site can accept any of the four canonical dNTP [Adapted from 159].*

It has been suggested that the active tetrameric form of SAMHD1 can persist for extended periods even after dNTP levels have diminished below the threshold for SAMHD1 activation [162, 168]. This long-lived active state may be important for maintaining cellular dNTP pools at the extremely low concentrations observed in no-cycling cells such as macrophages and resting CD4+ T lymphocytes. The fine-tuned autoregulatory mechanism enables SAMHD1 to sense small fluctuations of dNTP concentrations within the cell and respond accordingly by degrading them to physiologically appropriate levels [167]. The degradation rate of each dNTP seems to be determined by its affinity for the active site and its intracellular concentration. However, recent studies suggest that the particular substrate bound into the RS2 affects dNTP specificity and catalytic efficiency [168].

Additionally, SAMHD1 is regulated through post-translational modifications such as phosphorylation at multiple sites [169, 170]. Phosphorylation at the C-terminal Tyrosine

592 residue (P-T592) of SAMHD1 is the most extensively studied [171, 172]. P-T592 of SAMHD1 take place during cell cycle by cyclin-dependent kinases 1 and 2 (CDK1/2) and coincides with an increase in intracellular dNTPs prior to S-phase DNA replication [173-176]. This phosphorylation likely occurs as cells emerge from the G_0/quiescent state and enter the G_1 phase [174]. Conversely, SAMHD1 predominates in a dephosphorylated state in non-cycling/quiescent cells, corresponding with reduced dNTP levels [172, 177, 178]. Phosphorylation negatively regulates SAHMD1 tetramerization and dNTPase activity [175, 179, 180], leading to the increment of intracellular dNTP pools [181] (**Figure 9**). In fact, it has been shown that the selective CDK4/6 inhibitor, palbociclib, blocks HIV-1 reverse transcription through the inhibition of CDK2 dependent SAMHD1 phosphorylation in human myeloid and lymphoid cells; by reducing intracellular dNTP pools [144, 182-184].

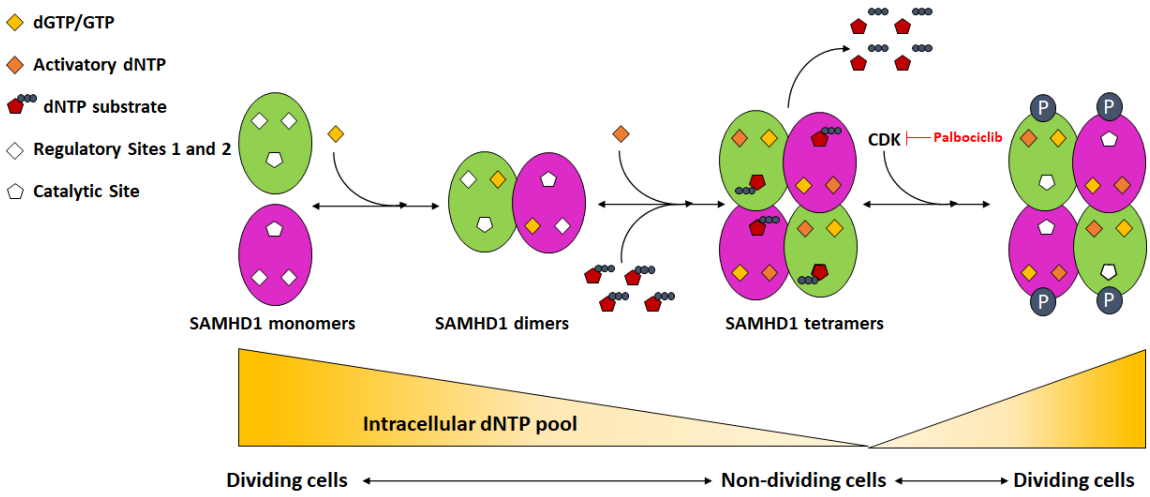


Figure 9. SAMHD1 regulation and dNTP homeostasis. *SAMHD1 catalytic activity is tightly controlled by regulatory nucleotides. Under conditions of low dNTPs, SAMHD1 exists in a monomer-dimer equilibrium. dGTP/GTP binding in RS1 stabilizes dimer conformation. Elevation of intracellular nucleotides results in dNTPs binding at RS2 and SAMHD1 tetramerization. Phosphorylation by CDKs destabilizes tetramerization without modifying catalytic efficiency, thereby allowing for an increase in dNTP pool necessary for DNA replication.*

7.1. The role of SAMHD1 in innate immunity, cell cycle and cancer

The functions of SAMHD1 go far beyond its role as a viral restriction factor. Mutations in SAMHD1 were first identified as being causative of Aicardi-Goutières syndrome (AGS) [185], a severe autoimmune disease caused by increased levels of IFN that manifests as an early-onset encephalopathy that usually, but not always, results in severe intellectual and physical disability.

AGS is an inherited encephalopathy characterized by the dysregulation of type 1 IFN responses and upregulation of interferon-stimulated genes (ISGs) putatively caused by dysregulation of nucleic acid metabolism. In addition of SAMHD1, mutations in other genes have been linled to AGS, including *TREX1, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, ADAR1, or IFI*H1 [186]. While more than half of the AGS patients exhibit abnormalities in cellular RNase H2 function, a small subset of patients has mutations in the *SAMHD1* gene [187]. AGS-associated mutations are found throughout the SAMHD1 gene and often lead to defects in its ability to oligomerize and therefore to maintain intracellular dNTP levels.

SAMHD1 also plays a crucial role in the maintenance of cell homeostasis through its dNTPase activity. SAMHD1 is ubiquitously expressed throughout all cell types. Also, its regulation is tightly synchronized with changes in dNTPs concentrations and cell cycle stages, as the maintenance of balanced intracellular dNTP pools are essential for genomic stability and appropriate DNA replication and repair. Thus, SAMHD1 is considered a central regulator of dNTP pool dynamics and its function can modify the replicative capacity of the cell [143, 149, 166, 185, 188]. Indeed, SAMHD1 has been considered as a tumour suppressor gene, due to its crucial role as a protector of genomic integrity and fidelity (**Figure 10**). On the other hand, dNTP pool imbalance due to deleterious mutations in SAMHD1 may eventually lead to a mutator phenotype and cancer [189-192]. Interestingly, several studies have associated SAMHD1 to lymphocytic leukaemia, lung adenocarcinoma, and colon cancer [193-196]. The Catalogue of Somatic Mutations in Cancer (COSMIC) has recorded 164 unique mutations to SAMHD1 found in samples obtained from various cancer tissues [124].

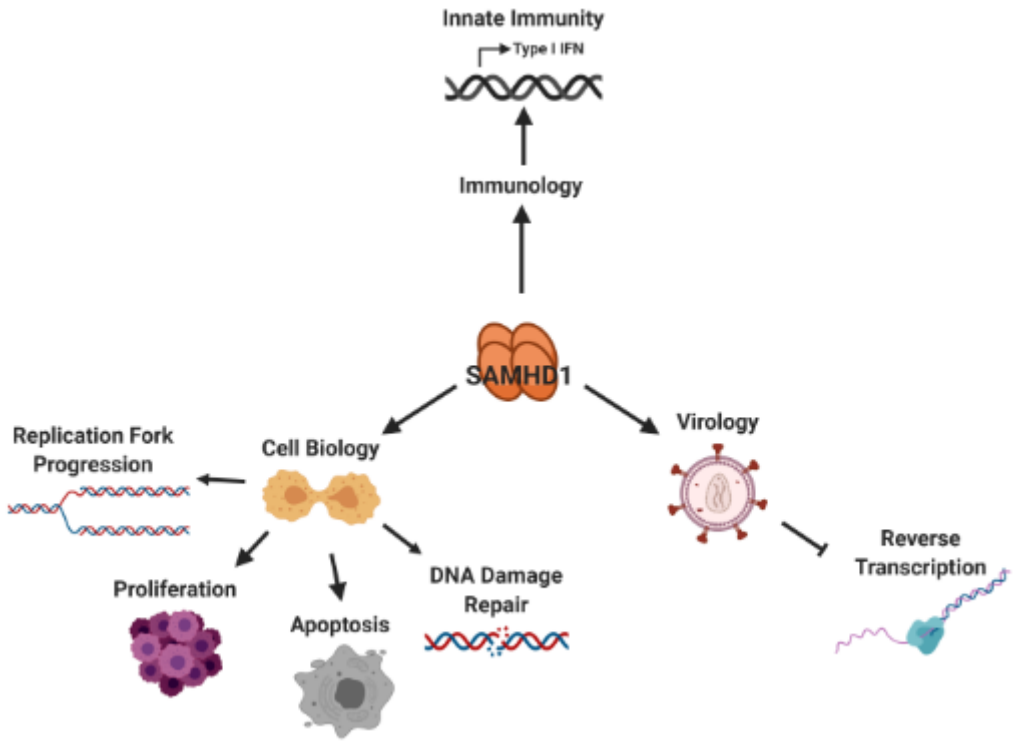


Figure 10. SAMHD1 roles in virology, immunology, and cell biology. *The dNTPase activity of SAMHD1 depletes intracellular dNTP pools in macrophages, restricting reverse transcription of HIV-1. In addition, SAMHD1 facilitates replication fork progression, is implicated in cell proliferation and apoptosis, and is localized to sites of DNA damage. As a negative regulator of INF I, SAMHD1 is commonly mutated in AGS syndrome [Adapted from 198].*

7.2. The role of SAMHD1 as a modulator of nucleotide analogue efficacy

SAMHD1 has also been recognized as a relevant factor that can modify the efficacy of nucleoside analogues, a type of drugs widely used as treatment for cancer and viral infections [199, 200, 267]. Following phosphorylation by intracellular kinases, nucleoside analogues are structurally similar to endogenous dNTP, and it has been shown that SAMHD1 could modify the efficacy of several of these analogues, either used as antiretrovirals [201-205] or as chemotherapeutic drugs [206-208]. Active SAMHD1 catalyses the hydrolysis and inactivation of a number of different nucleoside analogues, both used as antivirals or as chemotherapeutics to treat cancer.

In the field of HIV, several works have demonstrated that SAMHD1 modifies the efficacy of analogues used to treat HIV, with varying potency and efficacy depending on the specific cell type assessed [202-204].

The most notable effect of SAMHD1 in the modification of nucleoside-analogue efficacy comes from chemotherapeutic drugs. Cytarabine (Cytosar-U®, Ara-C), is the first line therapeutic agent for acute myelogenous leukaemia (AML). Nowadays, overwhelming evidence exists that demonstrate the key role of SAMHD1 in Ara-C efficacy both *in vitro* and *in vivo*. On one hand, SAMHD1 exhibits Ara-CTPase activity *in vitro* (285). Moreover, degradation or inactivation of SAMHD1 through genetic depletion, mutational inactivation of its triphosphohydrolase activity or proteasomal degradation using Vpx-expressing virus-like particles, potentiates the cytotoxicity of Ara-C in AML cells. Moreover, SAMHD1 expression levels negatively correlate with Ara-C treatment success in individuals with AML [207, 208, 212]. Thus, it has been proposed that SAMHD1 could be a potential biomarker for the stratification of patient sensitivity to Ara-C and that targeting SAMHD1 with Vpx could be an interesting therapeutic strategy to potentiate Ara-C efficacy in hematological malignancies [207, 213].

SAMHD1 has also been demonstrated to modulate clofarabine-induced toxicity in THP-1 and Hut-78 cells. Also, a significant negative correlation was observed between SAMHD1 expression and clofarabine-induced cytotoxicity in a panel of 133 haematological and lymphoid tissue-derived cell lines. Additionally, increased sensitivity to vidarabine, nelarabine, fludarabine, decitabine and trifluridine in SAMHD1 knock-out cells has been reported, suggesting that triphosphate variants of these drugs could be substrates for SAMHD1. [209-211].

Understanding the mechanisms of SAMHD1 modulation of drug efficacy could bring new insights into antiviral and anticancer therapy. Moreover, it is still necessary to evaluate the variety of compounds whose activity can be modified by the presence of SAMHD1 and the molecular interactions that take place. Furthermore, a deeper comprehension of cellular factors involved in the establishment and maintenance of HIV-1 latency is essential to ease the way for the development of a definitive curing strategy against HIV.

HYPOTHESIS AND OBJECTIVES

The complex nature of HIV/AIDS pathogenesis will require the development of combined therapeutic strategies to achieve an optimal and definitive cure for the diseases. Two major roadblocks exist in the finding of an HIV-1 cure and to improve the quality of life of people living with HIV (PLWH).

First, although ART suppresses HIV to undetectable levels, interrupting ART causes the virus to rapidly rebound and HIV-infected individuals must commit to lifelong ART to keep HIV replication suppressed, which does not fully prevent pathology. These limitations are due to drug side effects and/or incomplete viral suppression, particularly in viral reservoirs. Thus, identifying a marker of latently infected cells is key to develop new therapeutic approaches to be used in combination with ART or LRAs for HIV eradication.

Second, lifelong ART in PLWH may cause the appearance of a wide range of comorbidities that include a higher incidence of cancer, opportunistic infections, and immune diseases due to undetectable ongoing replication, drug side effects and chronic immune activation which leads to T cell exhaustion. The role of SAMHD1 as a restriction factor and immune modulator, and its capacity to modify nucleotide analogue efficacy might be of great importance in the generation of new therapeutic strategies not only for the treatment of HIV, but also in cancer and other infections.

Hence, the **main objective** of the present thesis is the evaluation and characterization of host factors that might be key for the progression and for the development of novel therapeutic strategies against HIV infection and cancer.

The **specific objectives** are the following:

1. To determine the value of CD32 as a marker of HIV latency.
    1.1. To evaluate the pattern of CD32 expression under different conditions of immune cell activation in acute and latent *in vitro* HIV infections.
    1.2. To evaluate the significance and contribution of CD32+ T lymphocytes in the maintenance of the replication-competent HIV-1 reservoir *in vivo*.

2. To explore the potential of SAMHD1 as a modifier of antiviral and anticancer therapeutic efficacy of nucleotide analogues and other antimetabolites.
    2.1. To determine the potency of SAMHD1 as a modulator of antimetabolite efficacy as antiviral and anticancer agents.
    2.2. To identify pharmacological modulators of SAMHD1 activity that improve antiviral and anticancer efficacy of antimetabolites.

MATERIALS AND METHODS

Primary cells

PBMCs were isolated from buffy coats of uninfected blood donors. The buffy coats were purchased from the Catalan Banc de Sang i Teixits (http://www.bancsang.net/en/index.html; Barcelona, Spain). The buffy coats were anonymous and untraceable, and the only information provided was whether they had been tested for disease. All donors provided informed consent at the time of blood extraction. Briefly, PBMCs were obtained using a Ficoll-Paque density gradient centrifugation and used for fresh purification of CD4+ T lymphocytes by the EasySep™ Human CD4+ T Cell Enrichment negative selection Kit (StemCell Technologies, catalog #19052). Purity of the populations was confirmed using flow cytometry.

Both isolated CD4+ T lymphocytes and total PBMCs were kept in complete Roswell Park Memorial Institute (RPMI) 1640 medium (Thermofisher/Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermofisher/Gibco), penicillin and streptomycin (Thermofisher/Gibco), with IL-2 alone (3 ng/ml, Sigma-Aldrich, ref. 011011456001) or IL-2 and one of the following stimuli PHA (4 µg/ml; Sigma-Aldrich, ref. L1668), anti-CD3 and anti-CD28 antibodies (Immunocult™, StemCell Technologies, ref. 10991) or IL-7 (5 ng/ml, Peprotech, ref. 200-07) when appropriate.

Monocytes were purified using negative selection antibody cocktails (#19359, StemCell Technologies), following the manufacturer protocol. Monocytes were cultured in complete culture medium, RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Gibco) and penicillin/streptomycin (Gibco) and differentiated to monocyte derived macrophages (MDM) for 4 days in the presence of monocyte-colony stimulating factor (M-CSF, Peprotech) at 100 ng/ml.

All protocols were approved by the Scientific Committee of Institut de Recerca de la Sida-IrsiCaixa and the Ethics Review Board of Hospital Germans Trias i Pujol.

Cell lines

The cell lines, source and culture conditions used in the present work are summarized in table 1. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) or RPMI (Gibco, Madrid, Spain) supplemented with 10% heat-inactivated FCS (Gibco, Life Technologies, Madrid, Spain) and antibiotics 100 U/ml penicillin, 100 µg/ml streptomycin (Life Technologies) and maintained at 37 °C in a 5% CO2 incubator.

Table 1. Cell line characteristics.

Cell line	Provider	Culture media
HEK293T	AIDS Reagent Program, National Institutes of Health, NIH, Bethesda, MD, USA.	DMEM +10% FCS +Pen/Strep
ACH2	AIDS Reagent Program, National Institutes of Health, NIH, Bethesda, MD, USA	RPMI +10% FCS+ pen/strep
TZM-bl	AIDS Reagent Program, National Institutes of Health, NIH, Bethesda, MD, USA	DMEM +10% FCS +Pen/Strep
MDA-MB-468 (ATCC® HTB132TM)	American Type Culture Collection, ATCC, Manassas, VA, USA	DMEM +10% FCS +Pen/Strep
T47D (ATCC® HTB-133TM)	American Type Culture Collection, ATCC, Manassas, VA, USA	DMEM +10% FCS +Pen/Strep

Patients and samples

HIV infected patients were recruited from the Infectious disease clinical unit of Hospital Germans trias i pujol. Patients were included if the individuals were older than 18 years old, had chronic HIV-1 infection and had previously been on highly active ART for >1 year. HIV-RNA levels were <400 copies/ml during at least 1 year and <50 copies/ml at study entry. Frozen PBMCs (isolated as described above for uninfected donors and stored in liquid nitrogen until used) or cells isolated from fresh peripheral blood from HIV+ individuals visiting our clinic were used for all determinations. All participants in the study provided informed consent, and the work was approved by the Scientific Committee of Fundació IrsiCaixa and the Ethics Committee of Hospital Germans Trias i Pujol. All methods were performed in accordance with relevant guidelines and regulations and the ethical principles suggested in the Declaration of Helsinki.

Virus and virus infections

Wild type NL4-3 plasmid expressing GFP (NL4-3-GFP) or modified to bind Vpx (NL4-3*GFP) were kindly provided by Dr. O. T. Keppler (Max von Pettenkofer Institute, Ludwig-Maximilians-Universität Munich, Germany) [221]. For obtaining viral stocks, wild-type plasmid NL4-3GFP or NL4-3*GFP together with the Vpx expression construct SIVmac239-plasmids were transfected with into HEK293T cells. Three days after transfection, the supernatants were collected, filtered, concentrated using Lenti-X concentrator (Clontech, ref. 631232) and stored at −80 °C.

To generate viral-like particles carrying Vpx (VLP-Vpx), HEK293T cells were co-transfected with pSIV3+ and a VSV-G expressing plasmid. Three days after transfection, supernatants were harvested, filtered, and stored at -80ºC.

The VSV-pseudotyped NL4-3-GFP virus, was obtained by cotransfection of an envelope-deficient HIV-1 NL4-3, clone encoding IRES-GFP (NL4-3-GFP) with VSV-G expression plasmid in HEK293T cells using polyethylenimine (Polysciences, Warrington, PA, USA) as previously described [201]. Viruses were titrated by infection of TZM cells followed by GFP quantification by flow cytometry.

Infection of CD4+ T lymphocytes was performed by spinoculation (1200 g, 2 h at 37 °C) in 96-well plates with 0.25×10^6 cells/well. After spinoculation, cells were kept in the incubator for 72 h prior to analysis by flow cytometry.

For MDMs infection, cells were pretreated with VLP-Vpx for 4h before infection or left with fresh media. After pretreatment with VLP-Vpx, cells were then infected with VSV-pseudotyped NL4-3-GFP and drugs were added at the time of infection. Viral replication was measured two days later by flow cytometry. The anti-HIV activity of the different compounds was determined by infection of cells in the presence of different concentrations of the corresponding drug and 50% effective concentrations (EC_{50}) were calculated, as previously described [202].

mRNA quantification

For relative mRNA quantification, RNA was extracted using the NucleoSpin RNA II kit (Magerey-Nagel, Cat num 740955) as recommended by the manufacturer, including the DNase I treatment step. Reverse transcription was performed using the PrimeScript™ RT-PCR Kit (Takara, Cat num RR036A). Gene expression levels of *FCGR2a* (CD32) were measured by a two-step quantitative RT-PCR and normalized to *GAPDH* mRNA expression using the ΔΔCt method. Primers and DNA probes were purchased from Life Technologies (TaqMan gene expression assays).

Integrated HIV-1 provirus DNA quantification

DNA was extracted using the DNA Quick extraction kit from Epicentre following the manufacturer's instructions. For integrated provirus DNA quantification, an LTR pre-amplification was performed to assure amplification of integrated HIV-1 only (forward 5'- GCCTCCCAAAGTGCTGGGATTACAG-3' or 5'-TGGCAGAACTACACACCAGG-3'; reverse 5'-TTGCCCATACTATATGTTTTAA-3') followed by quantitative PCR amplification of an internal LTR fragment using the following primers and probe: forward 5'-GACGCAGGACTCGGCTTG-3', reverse 5'-ACTGACGCTCTCGCACCC-3', and probe FAM 5'-TTTGGCGTACTCACCAGTCGCCG-3' TAMRA. Absolute quantification was obtained by extrapolating Ct data with a standard curve performed in parallel with a series of samples of known HIV-1 copy number, based on the ACH2 cell line.

Flow cytometry

For the characterization of PBMCs from uninfected donors and HIV+ individuals, cells were labelled with distinct antibodies (Table 2) that allowed to identify the different immune cell subpopulations. Cell doublets were removed from the analysis (FSC-A versus FSC-H) and lymphocytes were gated by using the forward and side scatter areas (FSC and SSC). In brief, monocytes were identified by staining with anti-CD3/anti-CD14 antibodies. T and B lymphocytes were stained using anti-CD3/anti-CD19 antibodies. Then, CD3+CD8+ double staining was used to identify CD4+ and CD8+ T lymphocytes

subpopulation. Anti-CD69, anti-HLA-DR antibodies were used to characterize the activation of CD4+ T lymphocytes and anti-CD32 antibody was used to identify CD32+ cells.

Cells were incubated with the different antibodies for 40 min at room temperature in the dark. Next, cells were washed with phosphate-buffered saline (PBS) and fixed with 1% formaldehyde (FA) prior to flow cytometry. For the determination of CD32 expression levels, an APC mouse IgG2b isotype control was included in parallel. Isotype positivity was set up at a threshold value of ≤0.1 in all cases. Flow cytometry was performed in a FACS LSRII flow cytometer (BD Biosciences). The data were analysed using the FlowJo software (Tree Star Inc., Ashland, OR).

Table 2. Antibodies used for the characterization of immune cell subpopulations in HIV+ individuals.

Antibodies	Cell type labeled	Provider
anti-CD3 PerCP	Human Lymphocytes	BD catalog # 340663
anti-CD8 BV510	Human CD8 Lymphocytes	BD catalog # 740175
anti-CD14-FITC/ PE	Human Monocytes	BD catalog # 347493/ 562334
anti-CD19-FITC	human B-lymphocytes	BD catalog # 347543
anti-CD69-BV421	Early CD4+ T lymphocytes activation marker	BD catalog # 562884
anti-HLA DR PeCy7	Late CD4+ T lymphocytes activation marker	BD catalog # 335795
anti-CD32-APC	monocytes/macrophages, B cells and T-lymphocytes	Sony Biotech, catalog # 2116040

For cell-sorting experiments, CD4+ T cells were labelled as described above for 40 min at 37 °C and kept in PBS supplemented with 1% FBS. The different CD4+ T cell subpopulations were identified by FACS, and the CD3+ CD8− CD14− population was sorted into the CD32+ or CD32− fractions using FACSAria II (BD Biosciences). For intracellular Ki67 staining, cells were fixed for 3 min with fixation buffer (FIX & PERM; Life Technologies Life technologies, cat num GAS004) before adding precooled 50% methanol for 10 min at 4 °C. Cells were washed in PBS with 5% FBS and incubated for 30 min with the Ki67 FITC Ab (1:10; clone B56; BD Biosciences, cat num 556026) diluted in permeabilization buffer.

Quantitative viral outgrowth assay

An ultrasensitive co-culture assay was applied to sorted CD32+ or CD32− CD4+ T cells isolated from a subset of HIV+ individuals on ART [230]. Briefly, purified cells (500–20,000 CD4+ cells) were stimulated with a pool of allogeneic irradiated PBMCs at a ratio of 1:5 with allogeneic PBMCs in 96-well plates in the presence of PHA (1 µg/ml) and IL-2 (100 U/ml) for 72 h and co-cultured for 7 days with a pool of stimulated CD8-depleted PBMCs from 3 HIV-seronegative donors. To maximize viral outgrowth during the following 2 weeks, the co-cultures were fed once a week with fresh medium and once a week with a pool of stimulated CD4+ cells from three HIV-seronegative donors. After 21 days in culture, the supernatants were assayed in CD4+ TZM-bl cells and the number of infectious units per million cells (IUPM) were calculated according to Rosenbloom et al. [214] with the use of the IUPM Stats v1.0 Infection Frequency Calculator (http://silicianolab.johnshopkins.edu). The use of reporter CD4+ TZM-bl cells has been shown to have a 1000-fold increase in sensitivity and helped demonstrate that the size of the inducible latent HIV-1 reservoir in aviremic participants on therapy may be ~70-fold larger than previous estimates [215].

Drugs

Small molecules specifically targeting different cellular or viral proteins were used to inhibit different molecular pathways.

Table 3. Compound list according to the drug type and their cellular or viral target.

Drug Name	Drug Type	Main Target	Provider
Zidovudine	NRTI	HIV reverse transcriptase	Sigma-Aldrich, Madrid, Spain
Nevirapine	NNRTI	HIV reverse transcriptase	Sigma-Aldrich, Madrid, Spain
Efavirenz	NNRTI	HIV reverse transcriptase	Sigma-Aldrich, Madrid, Spain
Cytarabine	Pyrimidine nucleoside analog	DNA synthesis	Sigma-Aldrich, Madrid, Spain
Nelarabine	Purine nucleoside analog	DNA synthesis	Sigma-Aldrich, Madrid, Spain
Cladribine	Purine nucleoside analog	Adenosine deaminase inhibitor	Sigma-Aldrich, Madrid, Spain
Clofarabine	Purine nucleoside analog	RNR; DNA polymerase	Sigma-Aldrich, Madrid, Spain
Floxuridine	Pyrimidine nucleoside analog	TS	Sigma-Aldrich, Madrid, Spain

Fluorouracil	Pyrimidine nucleoside analog	TS	Sigma-Aldrich, Madrid, Spain
Gemcitabine	Pyrimidine nucleoside analog	DNA synthesis, RNR, TS	Sigma-Aldrich, Madrid, Spain
Pemetrexed	Anti-folate	TS, dihydrofolate reductase, glycinamide ribonucleotide formyltransferase	Sigma-Aldrich, Madrid, Spain
Methotrexate	Anti-folate	Dihydrofolate reductase, TS, PURH	Eurodiagnosticos SL, Madrid, Spain
Palbociclib	Selective Cyclin-dependent kinase inhibitor	CDK 4/6	Selleckchem (Munich, Germany)
Ribociclib	Selective Cyclin-dependent kinase inhibitor	CDK 4/6	Selleckchem (Munich, Germany)
Abemaciclib	Selective Cyclin-dependent kinase inhibitor	CDK 4/6	Selleckchem (Munich, Germany)
Midostaurin	Multitarget kinase inhibitor	Multiple protein kinases involved in cell growth(*)	Sigma-Aldrich, Madrid, Spain

NRTI: Nucleoside analog reverse-transcriptase inhibitors; NNRTI: Non-nucleoside analog reverse-transcriptase inhibitors; RNR: Ribonucleoside-diphospate reductase, TS: Thymidylate synthase; PURH: Bifunctional purine biosynthesis protein; CDK4/6: Cyclin-dependent kinase 4/6; (*)PKCα/β/γ, Syk, Flk-1, Akt, PKA, c-Kit, c-Fgr, c-Src, FLT3, PDFRβ andVEGFR1/2.

Western Blot

Cells were rinsed in ice-cold phosphate-buffered saline (PBS) and extracts prepared in lysis buffer (50 mM Tris HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 10 mM Na β-glycerophosphate, 50 mM NaF, 5 mM Na Pyrophosphate, 270 mM sucrose and 1% Triton X-100) supplemented with protease inhibitor (Roche, Basel, Switzerland) and 1 mM phenylmethylsulfonyl fluoride. Lysates were subjected to SDS-PAGE and transferred to a PVDF membrane (ImmunolonP, Thermo, Waltham, MA, USA). The following antibodies were used for immunoblotting: anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:5000; Pierce,Waltham, MA, USA; anti-human Hsp90 (BD Biosciences, USA ref.610418), anti-SAMHD1 (1:2500; ab67820, Abcam, Cambridge, UK), anti-Rb (9309), anti-phospho-Rb (Ser807/811, 9308, anti-phospho-SAMHD1 Thr592 (15038) (all 1:1000; Cell Signaling Technologies, USA).

Evaluation of cytotoxicity

Drug cytotoxicity was evaluated by measuring cell viability in treated vs. untreated conditions. Cells were treated at the indicated doses of the test compounds for 3 days and the number of viable cells was measured by a tetrazolium-based colorimetric method (MTT method) as described elsewhere [283, 284]. The MTT assay measures the metabolic activity of cells, resulting in an extremely sensitive procedure to evaluate cell viability and cell proliferation, including the effect of cytostatic agents that slow or stop cell growth.

Evaluation of drug combinations

Drug combinations were evaluated using the combination index (CI)-isobologram equation, a method widely used in pharmacology to study drug interactions. Relative values of drug activity were used to calculate CI as implemented in the Compusyn software (Combosyn Inc., Paramus, NJ, USA). In brief, combination experiments were performed by using serial dilutions of each drug alone or a mixture of the two drugs evaluated, as recommended by the Chou–Talalay method using a non-constant ratio combination [216]. CI was calculated for all combinations and those combinations, including concentrations of SAMHD1-activating drugs around calculated IC50, were considered for quantification of drug combination effect. Drug combinations with CI < 1 were considered synergic.

Immunohistochemistry

Tissue sections from lung and pancreas tumor tissues (T2235188-1 and T2235152, respectively, Amsbio UK), were used to evaluate SAMHD1 expression in tumor tissues. All immunohistochemical analyses were performed at the histopathology core facility at Germans Trias I Pujol Research Institute. A polyclonal rabbit anti-SAMHD1 antibody (cat. no. 12586-1-AP, Proteintech, Rosemont, IL, USA) and an automated detection system were utilized. The specificity of the polyclonal antibody was previously tested by western blot analysis in cell lines and by immunohistochemistry using paraffin-

embedded normal tissue. Images were obtained in a Zeiss Axioskop 2 microscope using ZEN blue 2011 software.

Statistical analyses

All experimental data are presented as the mean ± standard deviation (SD), p-values were calculated using an unpaired, two-tailed, *t*-student test with the GraphPad PRISM software (GraphPad Software, San Diego, CA, USA).

RESULTS

CHAPTER 1. EVALUATION OF THE ROLE OF CD32 AS A MARKER OF THE HIV-1 RESERVOIR

Summary

HIV-1 infects activated CD4+ T cells and results in active viral replication or silent integration. Latency is established within a narrow time window after activation or during the transition of these HIV-infected and activated cells to resting memory CD4+ T cells. Cells latently infected with HIV-1 are not thought to produce viral proteins and have long been considered indistinguishable from uninfected cells for all practical purposes. Molecular signatures that allow for the identification of resting, latently infected cells would facilitate the study of HIV latency and accelerate the generation of new insights and therapeutic approaches. Recently, CD32, an Fcγ receptor not normally expressed on T cells, has been shown to be preferentially expressed in latently HIV-1-infected cells in an *in vitro* model of quiescent CD4+T cells that, if confirmed, will represent an excellent diagnostic tool [83]. Therefore, the precise role of CD32 as a marker of HIV latency must be carefully evaluated. Here we characterized the pattern of expression of CD32 using both, *in vitro* models of acute and latent HIV infection, and samples of HIV+ individuals. Furthermore, we determined the enrichment of integrated HIV DNA in CD4+ T cell subpopulations to evaluate the contribution of these cells to the HIV reservoir. Additionally, we determine the potential of CD32 as a marker a replication-competent HIV-1 reservoir.

1.1. *In vitro* evaluation of CD32 as a marker of latently HIV-infected cells

CD4+ T-cell activation is thought to be a major factor in facilitating HIV-1 infection [219, 220]. Conversely, in resting CD4+ T cells, HIV is unable to achieve a productive infection due to restriction mediated in part by SAMHD1 [228]. Thus, latently infected CD4+ T lymphocytes are considered to be in a resting, non-proliferative and non-activated state. Hence, to characterize the expression patterns of CD32, we first focused on the study of well-stablished T-cell activation signatures, such as the early CD69 and the late HLA-DR activation markers [217, 218] in the CD32+CD4+ cell population, both in uninfected and in *in vitro* HIV infected primary CD4+ T cells.

1.1.1 Evaluation of CD32 expression in uninfected lymphocytes

In order to accomplish our first objective, we evaluated CD32 expression in purified uninfected primary CD4+ T cells from donor PBMCs under different activation stimuli *in vitro*. Primary CD4+ T cells were incubated for 3 days with different stimuli including PHA/IL-2, anti-CD3/CD28 antibodies and IL-2, IL-7 and IL-2, IL-2 alone or without any stimuli.

Expression of CD32 and the activation markers CD69 and HLA-DR were measured by flow cytometry. For this purpose, a gating strategy was set up to specifically identify CD4+ T lymphocytes. CD4+ T cells were defined as CD3+/CD8- cells. The presence of conjugates between T cells and cells known to express high levels of CD32, such as CD19+ B cells or CD14+ monocytes [222], was excluded by gating on forward scatter (FSC) singlets and measuring the expression of CD19+ and CD14+ in the CD32+ cells and/or the CD4+ T-cell population (**Figure 11**).

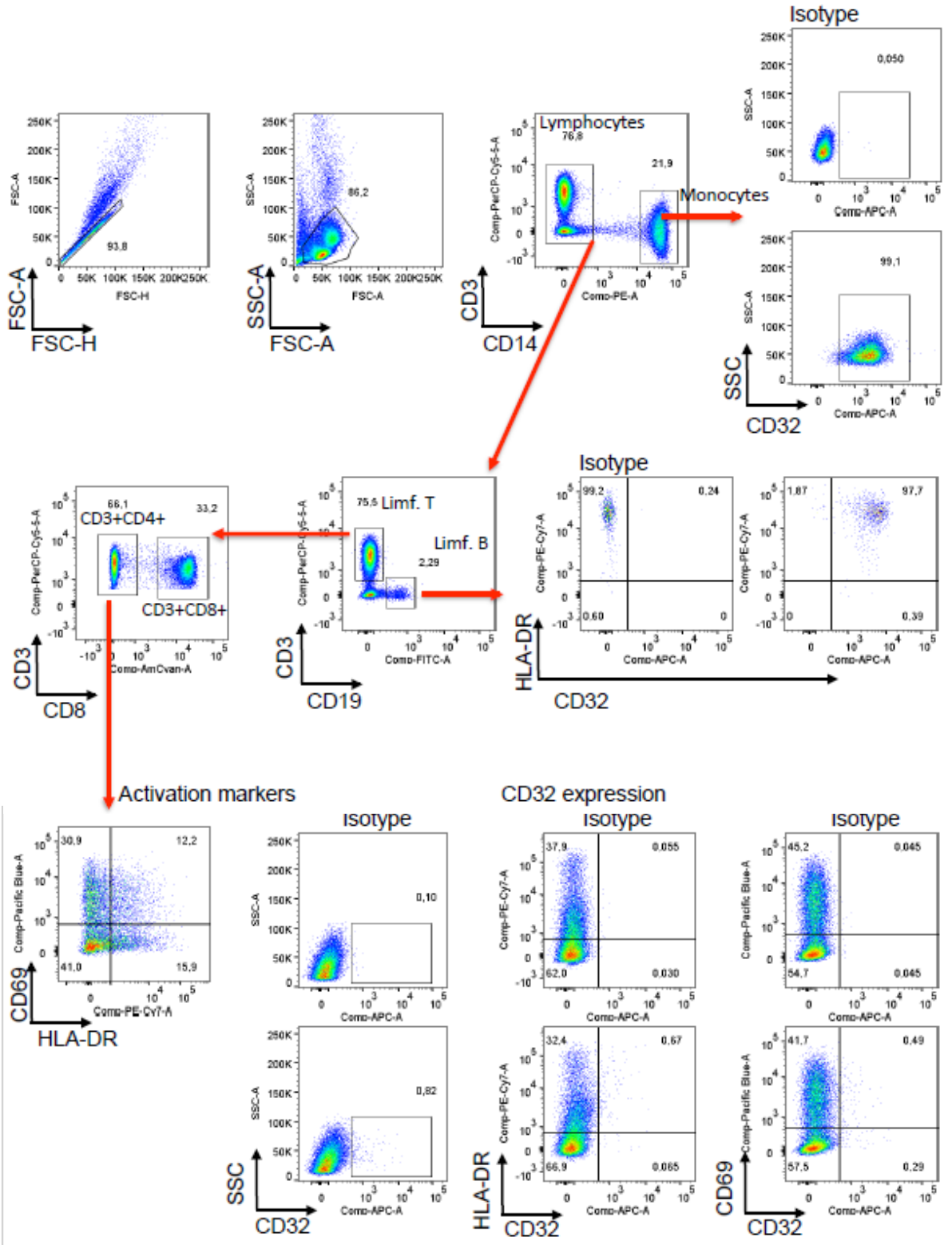


Figure 11. Gating strategy in the IL-2, PHA/IL-2 and anti-CD3/CD28 treated PBMCs or CD4+ T cells from donors. *Cell doublets were removed from the analysis (FSC-A versus FSC-H) and lymphocytes were gated by using the forward and side scatter areas (FSC and SSC). Monocytes (upper/right) and B lymphocytes were excluded by labelling CD14 and CD19 cell surface markers. The marginal CD8+ T cell population found after negative selection was excluded and cell activation markers (HLA-DR and CD69) were measured in the CD4+ population in combination with CD32. Dot plots from a representative donor are shown.*

Stimulation with IL-2, PHA/IL-2, anti-CD3/CD28/IL-2 and IL-7/IL-2 induced CD32 expression as measured by flow cytometry, with a 15-fold increase ($p<0.0005$) when stimulated with anti-CD3/CD28+IL-2 (**Figure 12** and **13A**). Additionally, to investigate the proliferation status and cell cycle, intracellular Ki67 staining was assessed at day 3 post stimulation. CD32 expression was significantly associated with cell proliferation as measured by intracellular Ki67 expression or T cell activation (**Figure 12** and **13B**). Up to 80-90% of total CD32+ cells were HLA-DR+ when stimulated with PHA/IL-2, anti-CD3/CD28/IL-2, and IL-7/IL-2, and up to 75-80% were CD69+ when stimulated with PHA/IL-2 or IL7-/IL-2 (**Figure 13C**). HLA-DR+ and CD69+ cells have upregulated CD32 expression compared with HLA-DR- and CD69-negative cells (**Fig 13D**). These findings clearly indicate that CD32 is expressed upon T cell activation.

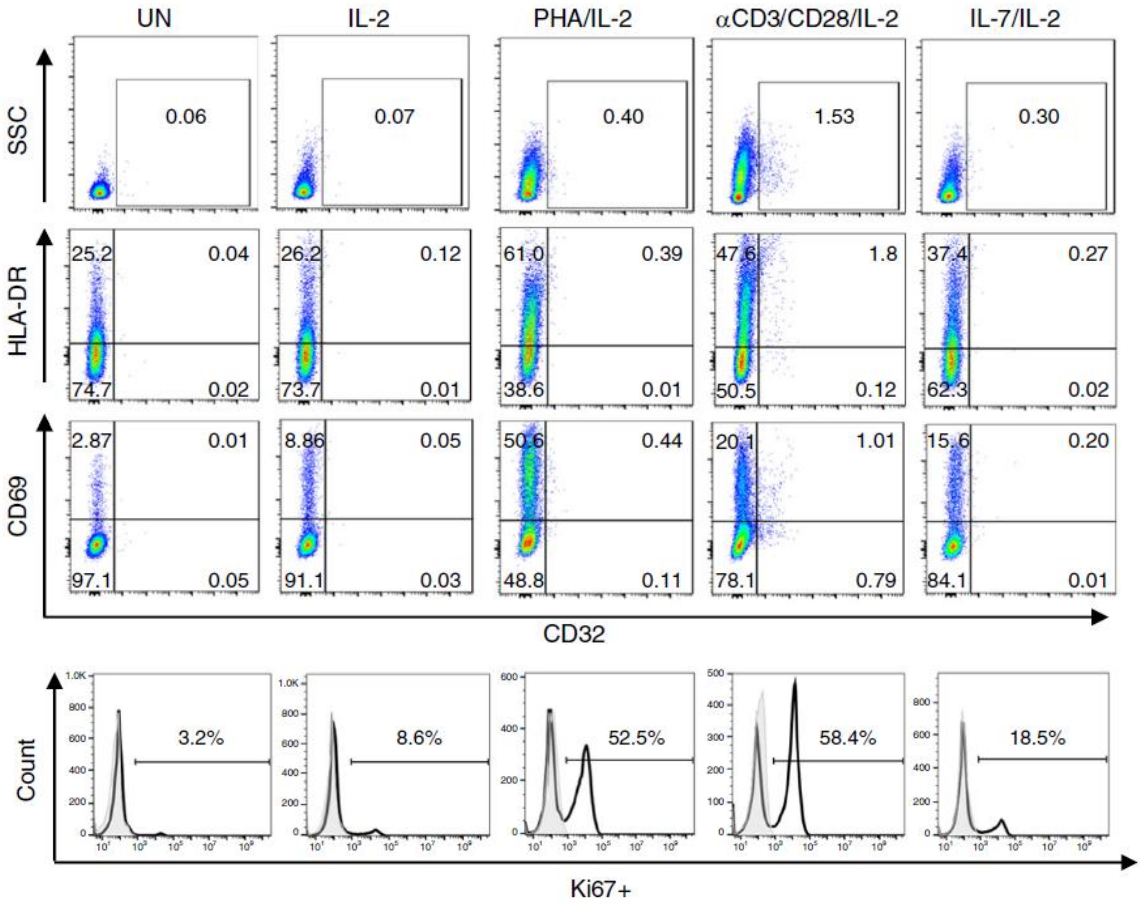


Figure 12. ***CD32 is a marker of T-cell activation.*** *Flow cytometry dot plots showing co-expression of CD32 and markers of cell activation and proliferation in unstimulated (UN) PBMCs or those stimulated with different stimuli. Dot plots of a representative donor is shown.*

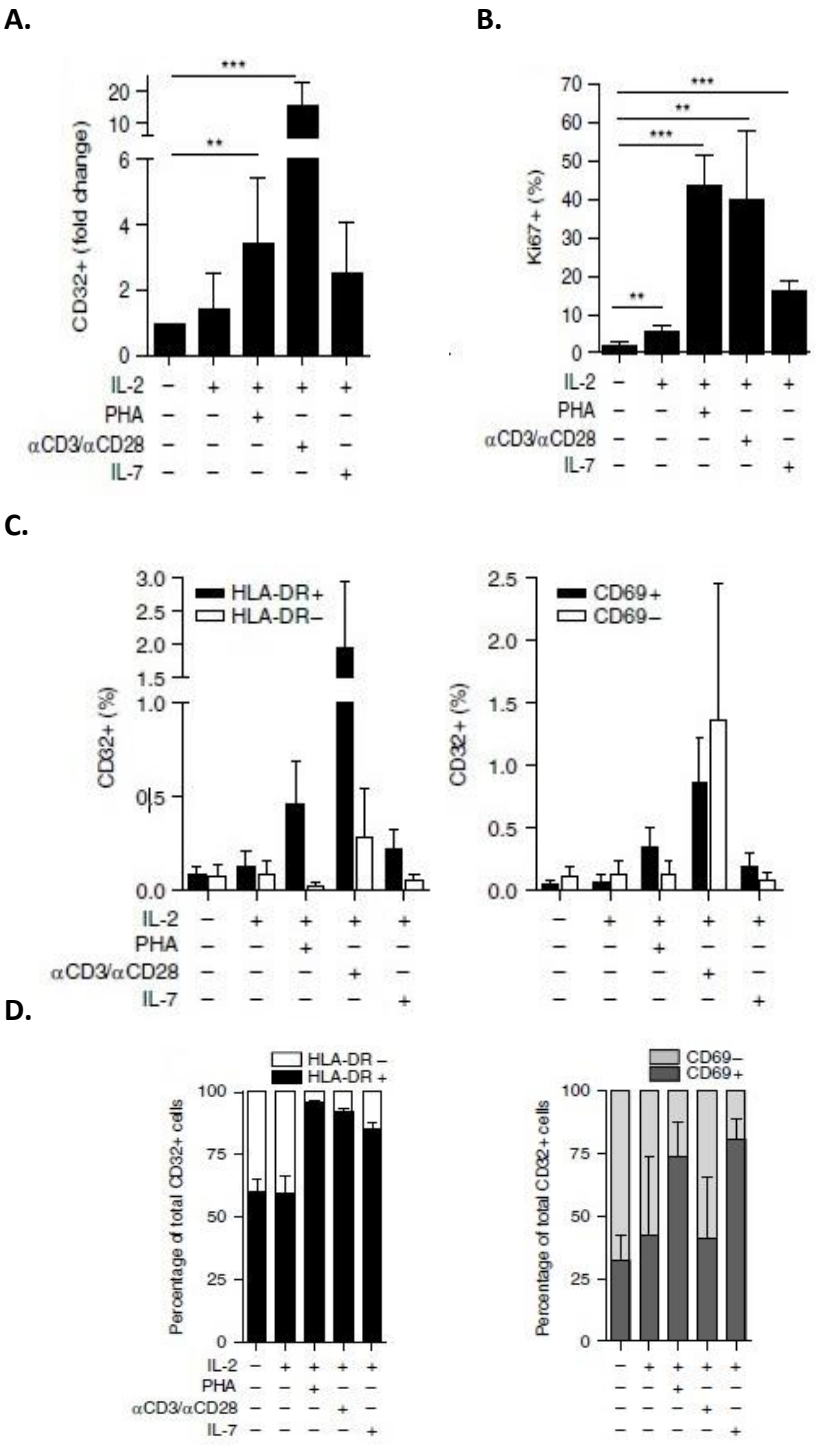


Figure 13. ***CD32 is a marker of T-cell activation. (A)*** *Fold change of CD32 expression in CD4+T cells unstimulated or stimulated with different conditions from uninfected donors. The cells were cultured in the presence of different stimuli for 72h, and protein levels of the cell surface marker CD32 were evaluated by flow cytometry. (**B**) Percentage of Ki67+ cells after activation with different stimuli. (**C**) Upregulation of CD32 correlates with the expression of activation markers HLA-DR and CD69 after activation. Relative contribution of HLA-DR (left panel) or CD69 (right panel) cells over the total population of CD32-expressing cells. (**D**) Individual data of HLA-DR cells (left panel) or CD69 (right panel) cells in the CD32 compartment. All panels represent the mean ± SD of at least five different donors. Student's t-test, *p<0.05, **p<0.005, ***p<0.0005.*

1.1.2 CD32 expression following HIV-1 infection

To determine the role of HIV-1 in CD32 expression, we evaluated cell surface marker expression following *in vitro* acute HIV-1 infection. CD4+ T cells were spinoculated with a wild type NL4-3-GFP HIV-1 virus and 72 hours later, CD32 expression was measured by flow cytometry. HIV-1 infection induced CD32 expression in PHA/IL-2 activated CD4+ T cells (**Figure 14A**). The effect was dependent on the multiplicity of infection used (**Figure 14B**) and was inhibited by the RT inhibitor efavirenz (**Figure 14C**), indicating that the effect was dependent on productive HIV-1 replication. However, only a small fraction of HIV-1+ cells were CD32+ (**Figure 14D**), and the ratio of HIV+ infected to uninfected cells did not significantly change depending on CD32 expression (18% vs. 16%, **Figure 14A**, right panel). This finding indicated that CD32+ cells were not preferentially infected compared with HLA-DR+ cells. These results are in line with the observation that most CD32+ cells are activated (HLA-DR+ and/or CD69+), but not all activated cells are CD32+ (**Figure 12**). Based on these results we can ascertain that productive HIV infection induce upregulation of CD32 although CD32+ cells are not preferentially infected.

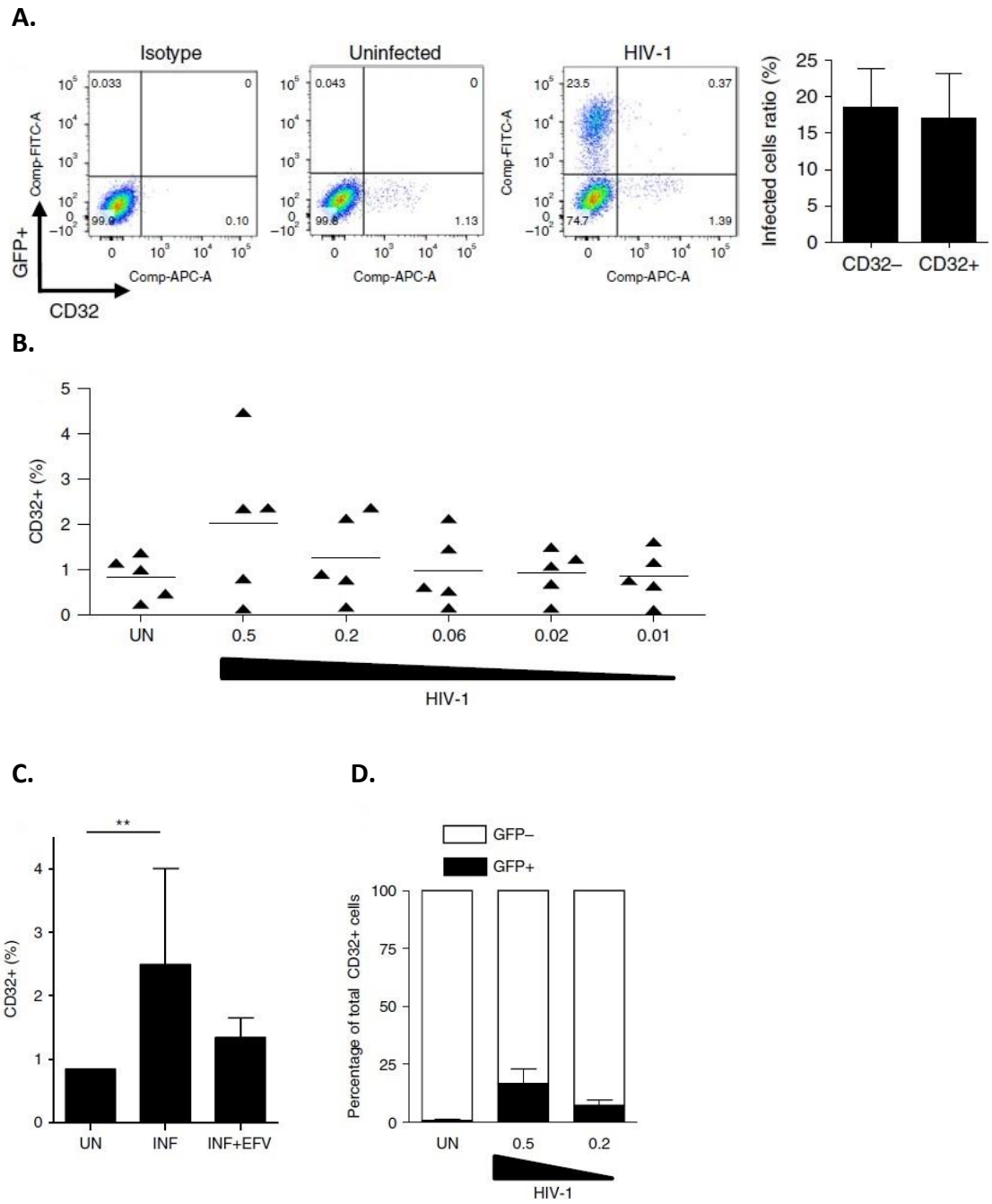


Figure 14. CD32 is upregulated after productive HIV-1 infection. ***(A)*** *CD32 expression in PHA/IL-2 stimulated CD4+ T cells infected with HIV-1 (a representative donor out of 5 is shown). The right panel indicates the ratio of HIV+ (GFP+) to CD32- or to CD32+ cells (N=5).* (***B)*** *Percentage of CD32 cell surface expression measured by flow cytometry and infected with different multiplicities of infection of HIV-1 NL4-3 or uninfected (UN).* ***(C)*** *Upregulation of CD32 expression after HIV-1 infection (INF) is reduced concomitant to blockade of HIV-1 infection with efavirenz (INF+EFV).* (***D)*** *The percentage of HIV-1+ (GFP+) cells in the CD32+ compartment. The data represent the mean ±SD of five different donors.*

An alternative strategy to evaluate HIV-1 infection and latency in CD4+ resting cells is to allow purified resting cells to be permissive for HIV-1 infection after degrading SAMHD1 [83], that is active in resting cells [145, 221]. Here, we recapitulated this strategy with an HIV-1 NL4-3 virus modified to incorporate Vpx into HIV-1 virions (HIV-1* Vpx GFP) [181, 202, 221] and effectively infect resting (IL-2 only) CD4+ T cells (**Figure 15A**). Infection with HIV-1* Vpx GFP-induced CD32 expression. The induction was dependent on the viral input (**Figure 15B**) and blocked by efavirenz (**Figure 15C**). After a 48h incubation, cells were sorted based on CD32 expression. The contribution of proviral DNA in CD32+ cells was evaluated by measuring integrated provirus DNA. We found more integrated DNA copies in the CD32- compartment in 4 out of 5 infected donor cells tested (**Figure 15D**). The preferential infection of CD32+ cells in one donor (D2) was associated with significantly higher CD4 T-cell activation as measured by HLA-DR and CD69 expression (**Figure 15E**), further indicating that CD32 expression is a marker of T-cell activation.

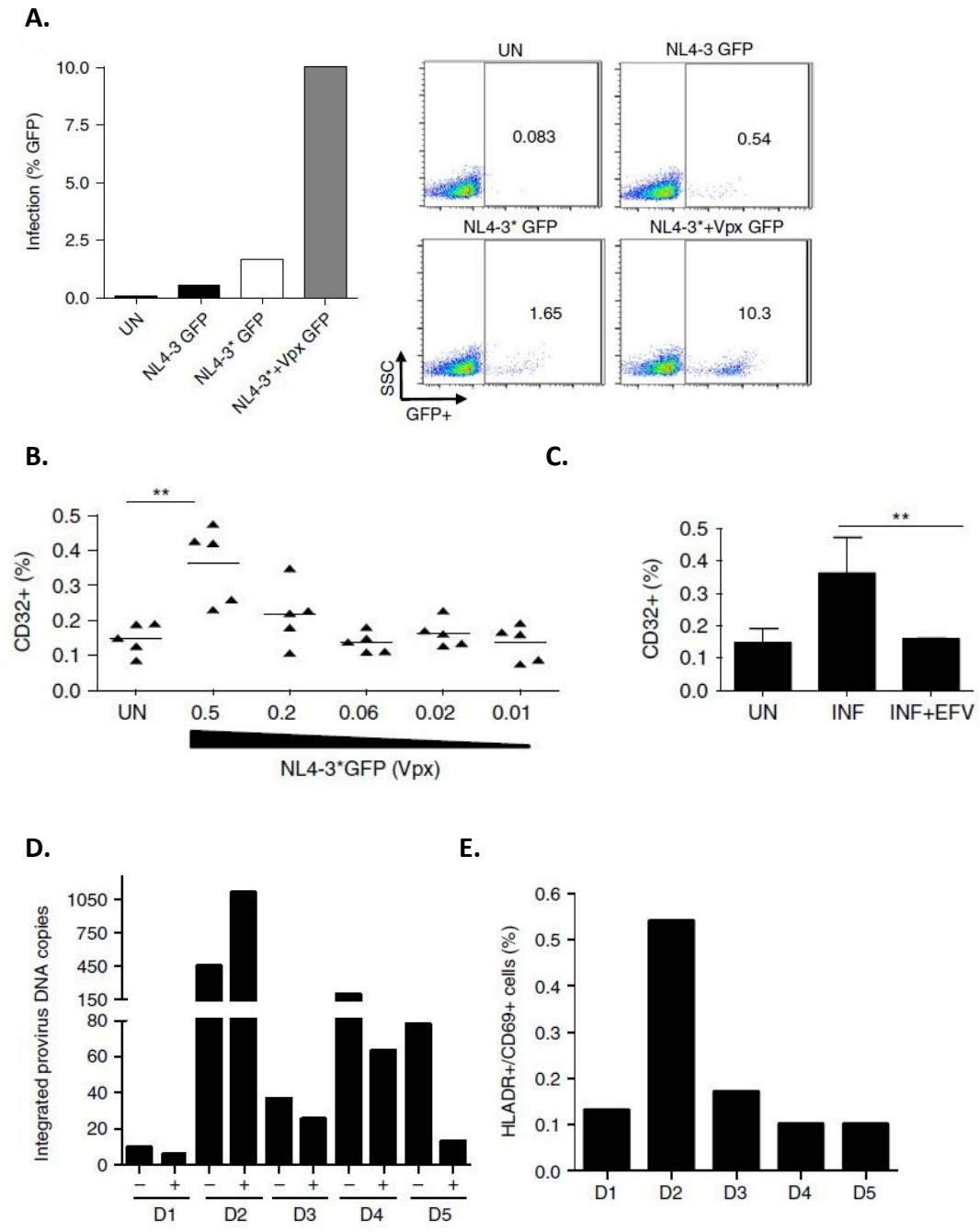


Figure 15. Contribution of HIV-1 proviral DNA in CD32+/CD4+ T cells from *in vitro* infections. ***(A)*** *Infection of CD4+ T cells treated with IL-2 and infected with NL4-3GFP, NL4-3*GFP and NL4-3*GFP carrying Vpx. The percentage of infection was evaluated using flow cytometry; representative dots are shown on the right. Data from a representative donor are shown. (**B**) Percentage of CD32 cell surface expression measured by flow cytometry and infected with different multiplicities of infection of HIV-1 NL4-3 carrying HIV-2 Vpx or uninfected (UN) (n=2). Lines represent mean values. **(C)** Upregulation of CD32 expression after infection (INF) is reduced concomitant to blockade of HIV-1 infection with efavirenz (INF+EFV). The data represent the mean ±SD of five different donors. For (**b**) and (**c**) Student's t-test, **p>0.005. **(D)** Integrated HIV-1 DNA copy number in sorted CD32+ and CD32- cells of five different donors infected with NL4-3*(Vpx). Measurement of integrated proviral DNA was performed by pre-amplifying an LTR DNA fragment with equal amount of genomic DNA input (100ng) from sorted CD32+ or CD32- population. Absolute quantification was obtained in a second amplification of HIV-LTR by qPCR. The data from each donor are shown. **(E)** Activation level of CD4+ T cells from five uninfected donors as measured as the expression of HLA-DR and CD69 cell surface markers by flow cytometry.*

In summary, these findings indicate that CD32 expression is mainly the consequence of T-cell activation induced either by exogenous stimuli or HIV-1 infection. However, we found no significant differences between the ratio of infected (GFP+) cells in CD32+ compared with CD32− cells, indicating that CD32 is not a preferential marker for infection, even though the majority of CD32+ cells also co-express the activation marker HLA-DR. Moreover, using a model of latent infection, the amount of HIV proviral DNA was higher in the CD32- population, strongly suggesting that CD32 is mainly a marker of T cell activation and not a marker of HIV latency.

1.2. Evaluation of CD32 as a marker of latently HIV-infected cells in patients

In the study published by Descours et al., CD32 expression was linked to latently HIV infection in patients under effective ART, by observing an enrichment (~1,000-fold) in HIV DNA in CD4+ T cells with high CD32 expression as compared to CD32-CD4+ T cells [83]. Thus, we aim to evaluate HIV proviral DNA in HIV-infected patients in order to comprehend the role of CD32 as a marker of HIV reservoir *in vivo*.

1.2.1. Characterization of CD32 expression in HIV+ patients under ART

CD4+ T cells from HIV+ individuals commonly express high levels of T-cell activation markers, even after effective ART [223-226]. Thus, once shown that CD32 is a marker of CD4+ T cells activation and that proviral DNA is not enriched in CD32+ population *in vitro*, we aimed to determine the role of CD32 expression and its relationship to HIV latency in samples from HIV+ individuals under effective ART. The immunological, virological status and combinatory treatment of all the patients are listed in table 4. All participants had viral loads under 50 HIV RNA copies/ml and the number of CD4+ and CD8+ cells were within the normal parameters at the time of sample collection (**Table 4**). The same gating strategy described in figure 11 was used and isotype control labelling was set to a stringent criterion (≤0.1% positive cells) to avoid overestimating CD32 expression. The 0.1% marker sets the boundary of three standard deviations of a standard Gaussian distribution or a common standard in flow cytometry [227].

CD32 expression was significantly higher in unstimulated CD4+ T cells from HIV+ individuals than uninfected, unstimulated donors ($p<0.001$, **Figure 16A**), and it was highly associated with the activation marker HLA-DR but not with CD69 (**Figure 16B, C**). This finding indicates a possible lack of functionality in T cells, since it has been shown that CD69 expression reliably predicts the anti-CD3-induced proliferative response of lymphocytes from HIV-1+ patients [228, 229]. A mean of 79.2% (70-94) of CD32+ cells were HLA-DR+ (**Figure 16D)** indicating a strong correlation between CD32 expression and T-cell activation.

Table 4. Immunological and virological characteristics of HIV+ individuals

Patient number	CD4 (cells/µl)	CD8 (cells/µl)	VL (copies/ml)	Treatment*
P1	1746	1474	<50	ABC,DDI,NVP
P2	607	638	<50	ABC,3TC,NVP,TDF
P3	877	1602	<50	D4T,IDV,3TC,RTV
P4	796	1263	<50	ABC,LPV,NVP,RTV
P5	984	1650	<50	DDI,EFV,D4T,3TC
P6	902	859	<50	EFV,3TC,AZT
P7	987	740	<50	D4T,IDV,3TC
P8	690	1074	<50	DDI,NVP,AZT
P9	935	1210	<50	IDV,3TC,AZT
P10	382	870	<50	ABC,3TC,RIL
P11	1340	1604	<50	DTG,MRV,RIL
P12	723	NA	<50	DTG,RIL
P13	234	620	<50	ELV,COBI,FTC
P14	279	NA	<50	DTG,RIL
P15	511	1235	<50	ABC,LPV,NVP,RTV
P16	719	464	<50	ELV,COBI,FTC,TAF
P17	340	1054	<50	EFV,TDF,FTC
P18	450	430	<50	DRV,COBI
P19	854	919	<50	ELV/C/F/TAF
P20	979	852	<50	RAL/TRU
P21	1240	897	<50	DTG,ABC,3TC
P22	897	481	<50	ELV/C/F/TAF
P23	943	849	<50	DTG,ABC,3TC

all values at the time of cell sample collection

*ABC abacavir, DDI didanosine, NVP nevirapine, 3TC lamivudine, TDF tenofovir, D4T stavudine, IDV indinavir, RTV ritonavir, LPV lopinavir, AZT zidovudine, EFV efavirenz, FTC emtricitabine, DTG dolutegravir, MRV maraviroc, RIL rilpivirine, ELV elvitegravir, COBI cobicistat, TAF tenofovir alafenide fumarate, DRV darunavir, VL HIV-1 plasma viral load, NA not available

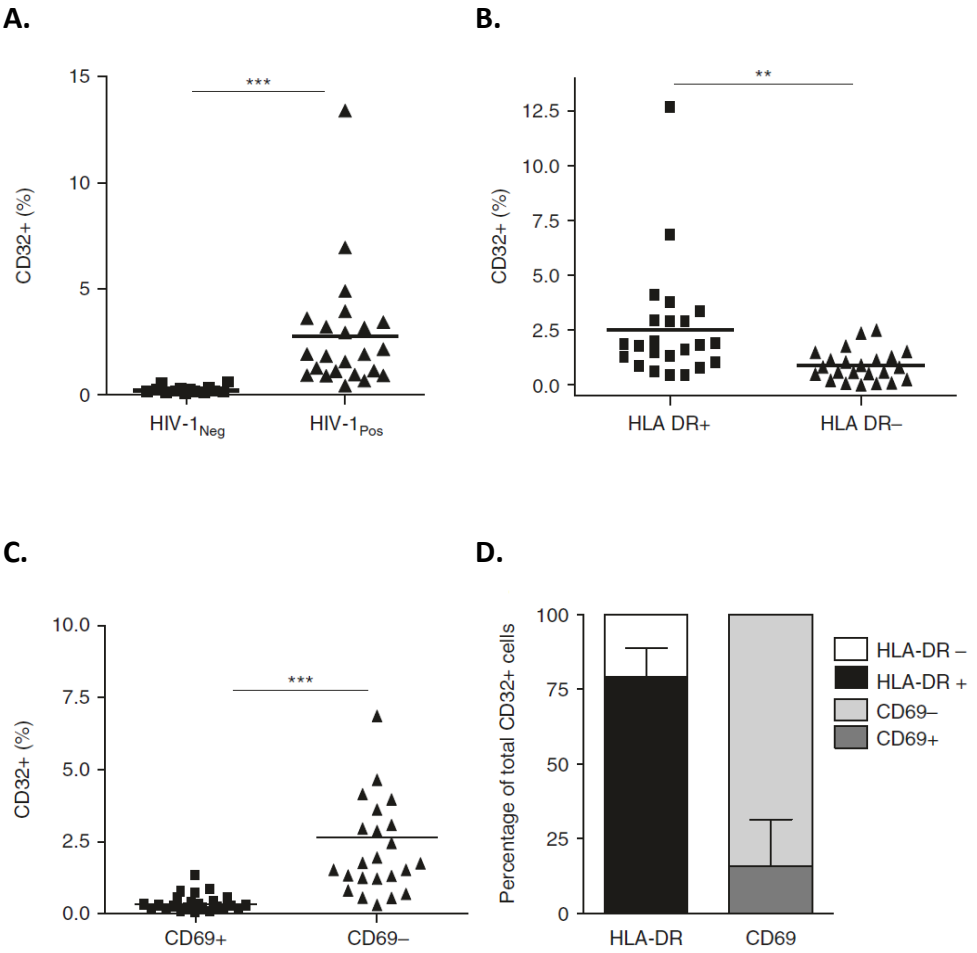


Figure 16. CD32 expression is associated to HLA-DR in CD4+ T cells from HIV-1+ individuals. ***(A)*** *Expression of cell surface CD32 measured by flow cytometry in CD4+ T cells from uninfected donors (HIV-1_{Neg}) and HIV-1+ individuals (HIV-1_{Pos}).* ***(B*** *and* ***C)*** *Percentage of CD32+ cells in HIV-1-infected individuals co-expressing HLA-DR* ***(B)*** *or CD69* ***(C)****. CD32 cell surface expression was measured in 23 HIV-1+ individuals by flow cytometry in combination with the HLA-DR (left panel) or CD69 (right panel) activation markers.* ***(D)*** *Contribution of the HLA-DR and CD69 cells to the CD32 compartment in CD4+ T cells from HV-1+ individuals. The data represent the mean ±SD from uninfected donors (N=14) and HIV-1+ individuals (N=23). Student's t-test, **p<0.01, ***p>0.001.*

1.2.2. CD32 expression does not correlate with integrated HIV-1 DNA

To determine whether CD32+ cells harbour more integrated HIV-1 DNA than CD32- cells, and thus, confirm the contribution of this cells to the viral reservoirs that remain stable over many years of ART, purified CD4+ T cells from 10 HIV-1+ individuals under ART were sorted using CD32 expression, and integrated provirus DNA was measured using qPCR. In 6 HIV+ individuals, integrated proviral DNA/cell was more prevalent in CD32- than CD32+ cells (**Figure 17A**).

However, there were no significant differences in the mean HIV-1 integrated proviral DNA/cell between the sorted populations (**Figure 17B**). In fact, the mean contribution of HIV integrated provirus DNA was significantly higher in CD32- than in CD32+ cells (p=0.017, **Figure 17C**), indicating that the vast majority of infected CD4+ T cells appear to be CD32-.

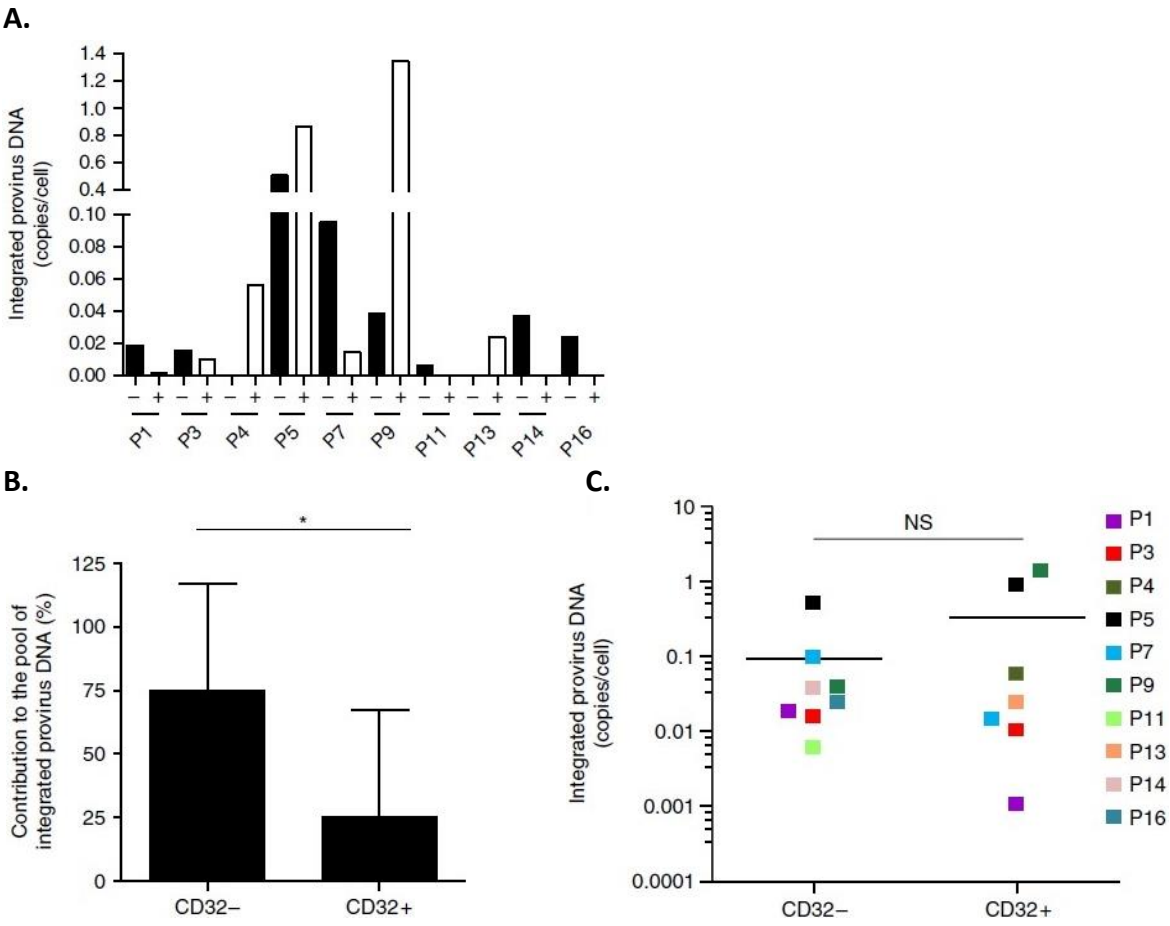


Figure 17. Contribution of HIV-1 proviral DNA in the CD32+/CD4+ T cells from HIV-1 individuals. ***(A)*** *Quantification of HIV-1 integrated provirus HIV-1 DNA copies per cell in CD32- (black bars) and CD32+ (white bars) CD4 T cells from 10 ART-treated individuals.* ***(B)*** *Individual HIV-1 integrated provirus DNA copies per cell in CD32- and CD32+ CD4 T cells. The mean values are presented as horizontal lines. Each color represents values from the same HIV+ individual. NS not significant (p=0.3).* ***(C)*** *Relative contribution of HIV-1 integrated provirus DNA in the CD32- and CD32+ CD4 T cell compartments from 10 ART-treated individuals. The mean values are presented as the percentage relative to the total number of integrated provirus DNA copies. Student's t-test, * p<0.05.*

1.2.3. CD32 does not mark a replication-competent HIV-1 reservoir

The most accepted definition of latent reservoir includes the requirement that cells infected with HIV must be able to reactivate and restart viral replication to induce a productive infection. Hence, to understand the significance and contribution of CD32+ cells in the maintenance of the HIV-1 reservoir, equal numbers of sorted CD32+ or CD32- CD4+ T cells from a subset of HIV-1+ participants were used to perform a quantitative viral outgrowth assay based on an ultrasensitive co-culture with stimulated donor cells for 21 days [230]. Co-cultured supernatants were titrated in CD4+ TZM-bl cells to evaluate the replication competence of the amplified virus, which was measured as luciferase production. In this model, released virus from CD32+ or CD32- CD4+ T cells should be competent enough to enter target cells and at least mediate Tat-dependent luciferase expression. There were no significant differences (Student's *t*-test, p=0.95) in the mean maximum likelihood estimate of infection frequency (in infectious units per million, IUPM) between CD32- and CD32+ cell cultures (**Table 5**). Excluding participants P15 and P22, in which virus outgrowth could not be determined for CD32+ cells, the estimated IUPM did not fall within the same order of magnitude for CD32- and CD32+ cells in only two comparisons: P5 and P23. This finding suggests that the virus that emerges after stimulation and co-culture of most of the HIV+ participant cells was similarly infectious regardless of CD32 expression.

Table 5. IUPM values for CD32- and CD32+ CD4+ T cells				
Participant	**CD32- CD4+ T cells**	**95% CI**	**CD32+ CD4+ T cells**	**95% CI**
P1	2039	762–5455	45,425	12,356–166,993
P2	5634	1266–20,245	8463	2452–23,579
P3	203,973	72,119–576,893	274,243	103,634–725,716
P5	6023	2684–13,514	10,414	2787–38,914
P7	277	103–740	888	419–1882
P9	10,207	2543–40,968	9049	2263–36,618
P15	11,556	2879–46,380	Und	-
P16	2253	1121–4523	2440	1209–4924
P19	482	117–1978	189	26–1344
P20	159,293	48,697–521,056	330,601	121,121–902,375
P21	189	26–1360	524	120–2271
P22	2835	654–12,279	Und	-
P23	128,163	68,593–239,465	7856	4698–13,119
MEAN±SD	40,994±71,810		42,401±69,174	

95% CI, lower bound and upper bound of 95% confidence interval, calculated according to Rosenbloom et al. [148]
IUPM infectious units per million cells, *Und* undetermined, *SD* standard deviation of the mean

In conclusion, we have evaluated the role of CD32 expression in HIV-1 infection, which has recently been proposed to be a marker of CD4 T cell HIV reservoir [83]. We found that CD32 expression is strongly associated with CD4+ T cells that co-express the activation markers HLA-DR and/or CD69 and correlates with cell proliferation (Ki67), concluding that CD32 expression is an activation marker of a subset of CD3+CD4+ T cells, as recently proposed [231]. Evaluation of cells from HIV+ individuals showed similar results, with ~90% of CD32+ CD4+ T cells co-expressing the activation marker HLA-DR.

Productive Infection of CD4+ resting T cells with a modified HIV-1 capable of overcoming SAMHD1-induced restriction, also shown increased CD32 expression. Indeed, proviral DNA in *in vitro* infected resting cells was preferentially found in the CD32− cells. Our data also challenge the robustness of CD32 as a marker of an HIV-1 reservoir. We found that in 6 out of 10 HIV+ individuals, the absolute contribution to the CD4+ T cell HIV-1 reservoir was higher in CD32− CD4+ T cells. In Descours et al. raw data show the same results, in which the absolute contribution of HIV-proviral DNA copies/ CD32+ cells were higher in only 5 of 9 HIV+ individuals, and in one case, the contribution was comparable between CD32− and CD32+ cells. Taken together, these data indicate that CD32+ cells are not a preferential HIV reservoir in all HIV+ individuals.

Thus, the role of CD32 in establishing an HIV-1 latent reservoir still requires further exploration and discussion because of its implications in designing therapeutic strategies for HIV. Confirmation of these findings could also redefine the concept of resting immune cells, not just for HIV.

The profound understanding of the complex mechanisms and molecular pathways involved in the induction and maintenance of HIV latency are critical to develop an effective therapeutical strategy. It has been proposed that the restriction factor SAMHD1 could play an important role in the regulation of viral silencing. SAMHD1 restricts HIV-1 replication in nondividing cells by degrading intracellular dNTPs and is highly expressed in resting CD4+ T cells, which are important for the HIV-1 reservoir and viral latency.

Additionally, it is becoming increasingly clear that modulation of SAMHD1 activity will be of great importance in the development of new insights in the treatment of infections and other diseases. By degrading cellular dNTPs, SAMHD1 plays a critical role in the homeostatic balance of cellular dNTPs and, thus, it may be a modulator of clinical efficacy of nucleotide-based treatments. Nucleotide metabolism plays a central role in cell proliferation, transformation, and tumour progression. Therefore, inhibition of nucleotide synthesis has been commonly used in the treatment of cancer, infectious diseases, and immune-mediated diseases [232].

CHAPTER 2. EXPLORING THE ROLE OF SAMHD1 AS A MODULATOR OF ANTIVIRAL AND ANTICANCER AGENTS

Summary

Sterile alpha motif and histidine-aspartic acid domain-containing protein 1 (SAMHD1) is a dNTP triphosphohydrolase involved in the regulation of the intracellular dNTP pool, linked to viral restriction, cancer development and autoimmune disorders. SAMHD1 function is regulated by phosphorylation through a mechanism controlled by cyclin-dependent kinases and tightly linked to cell cycle progression. Recently, SAMHD1 has been shown to decrease the efficacy of nucleotide analogues used as chemotherapeutic drugs. Thus, the second chapter of this thesis is dedicated to exploring the potential of the modulation of SAMHD1 activity in antimetabolite-based therapies to generate new therapeutical approaches in antiviral and anticancer treatments. For this purpose, we carried out a screening of the antiviral activity of a panel of antimetabolites currently used in cancer therapy in the presence or absence of SAMHD1, to determine the capacity of SAMHD1 to modify its activity and to understand the enzymatic mechanisms underneath. Further, taking into account the CDK-dependent regulation of SAMHD1 function, we developed an anti-HIV-1 assay to assess the potential of CDK inhibitors to boost efficacy of antimetabolites in antiviral and anticancer therapy, providing functional proof of the molecular pathways involved. Additionally, we evaluated SAMHD1 expression in different cancer tissues to identify those cancer types that could benefit from the pharmacological modulation of SAMHD1 function.

2.1. SAMHD1 determines the antiviral activity of several antimetabolites used in cancer therapy

The identification of SAMHD1 as a modulator of the anti-HIV activity of several nucleoside reverse transcriptase inhibitors (NRTI) [202] and more recently, of the chemotherapy agent cytarabine (AraC) [207], has opened the door to the possibility of evaluating its potential to improve drug efficacy of different antivirals and chemotherapeutic agents. Thus, in the second chapter of this thesis, we developed an antiviral assay in monocyte-derived macrophages (MDMs) to evaluate the value of SAMHD1 as a modulator of anticancer drug efficacy, including nucleotide analogues, but also anti-folate drugs and CDK inhibitors.

2.1.1. SAMHD1 regulates antiviral efficacy of antimetabolites in primary cells

Primary MDMs are susceptible to HIV-1 infection, and its replication capacity is dependent on SAMHD1 expression. Additionally, M-CSF-induced differentiation initiates MDM proliferation accompanied by SAMHD1 inactivation through phosphorylation mediated by CDK. Thus, HIV-1 infection of MDM provides an excellent model in which to test the activity of antimetabolite drug efficacy.

First, anti-HIV-1 activity of a panel of antimetabolite drugs used in cancer treatment was evaluated in MDMs in the presence or absence of SAMHD1. Cells were infected with VSV-pseudotyped NL4-3-GFP and drugs were added at the time of infection. Viral replication and cell viability was measured two days later by flow cytometry, by determining the percentage of GFP+ cells and gating live vs. dead cells, respectively. SAMHD1 degradation was achieved after transducing cells with HIV-2 Vpx, as confirmed by western blot (**Figure 18A**). Additionally, an increase in the percentage of HIV infection was observed in Vpx treated-MDMs, due to the replenishment of intracellular dNTP pool to the threshold required for retrotranscription.

As previously observed, Vpx-mediated degradation of SAMHD1 reduced the antiviral potency of the NRTI, AZT, compared to untreated macrophages, but did not change the activity of NVP, a non-nucleoside reverse transcriptase inhibitor (NNRTI) (**Figure 18B**). Conversely, degradation of SAMHD1 improved the anti-HIV-1 potency of AraC in MDM (**Figure 18C**).

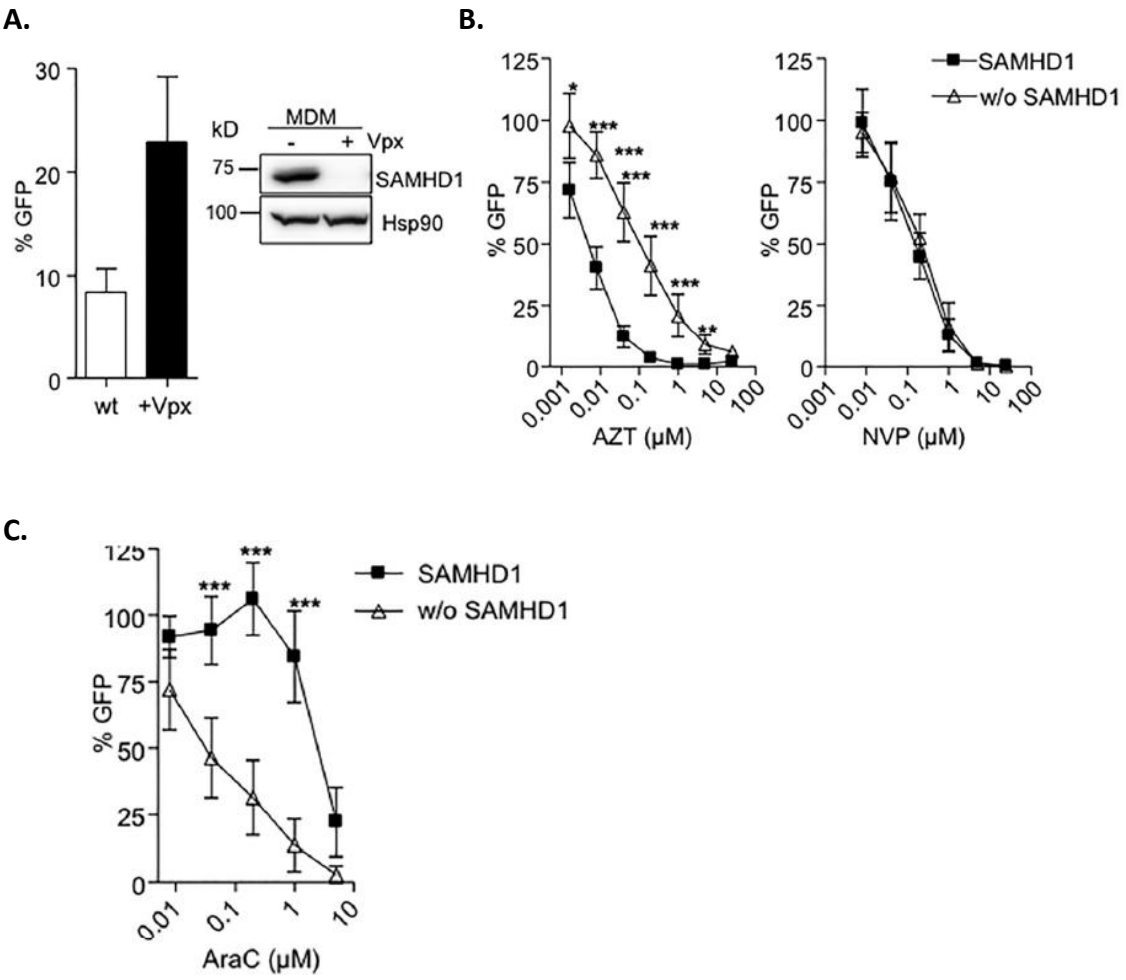
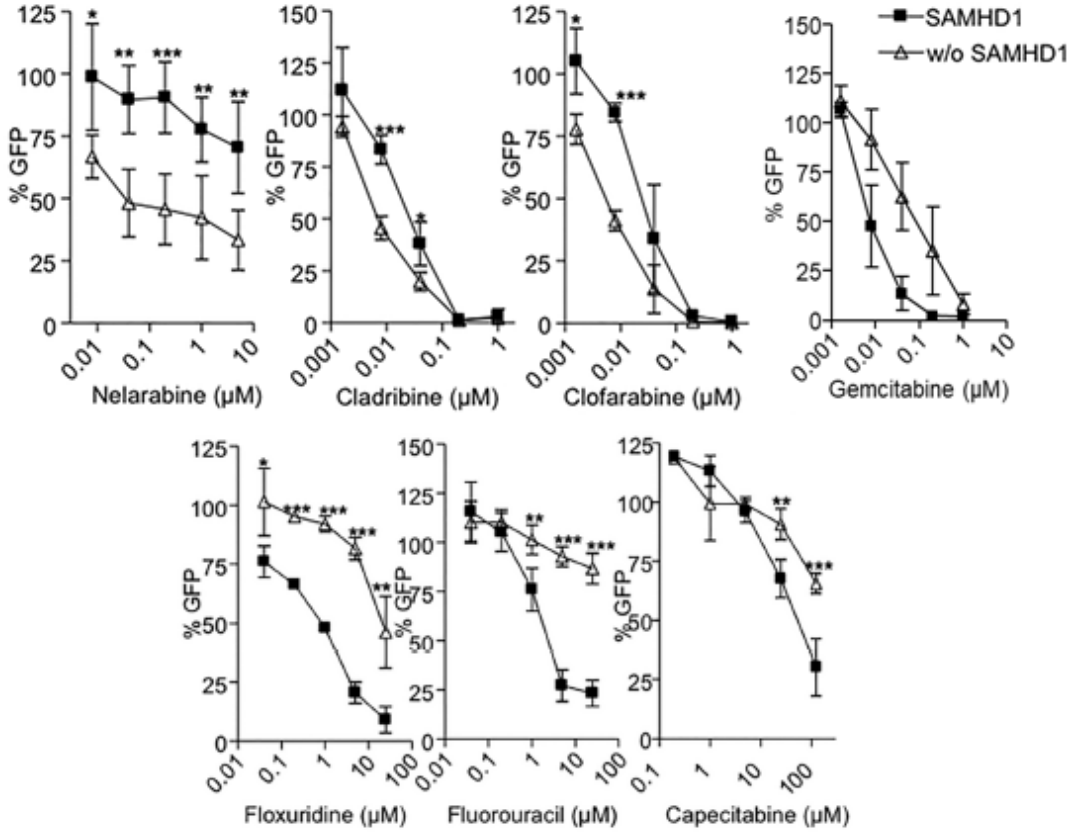


Figure 18. Degradation of SAMHD1 by HIV-2 Vpx enhances HIV-1 replication in MDMs. ***(A)*** *Cells previously treated or not with Vpx were infected with a VSV-pseudotyped HIV-1 GFP virus and replication was assessed two days later by measuring GFP expression. A 5-fold change in HIV-1 replication was observed after Vpx-mediated SAMHD1 degradation. Mean ±SD of ten independent donors performed in duplicate is shown. A representative western blot showing degradation of SAMHD1 expression in MDMs after Vpx treatment is shown. (**B**) Decreased sensitivity of AZT after Vpx-mediated SAMHD1 degradation in MDMs. Dose response of the NRTI AZT and NNRTI NVP, in wild type (■) or SAMHD1-depleted (Δ) MDM. Inhibition of HIV infection was measured as the percentage of GFP+ cells relative to the no drug condition. Mean ±SD of at least ten independent donors performed in duplicate is shown. (**C**) SAMHD1 modifies antiviral activity of AraC. Dose response of the AraC in wild-type (■) or SAMHD1-depleted (Δ) MDM. Inhibition of HIV infection was measured as the percentage of GFP+ cells relative to the no drug condition. Mean ±SD of at least three independent donors performed in duplicate is shown. Mean ±SD of at least three independent experiments performed in triplicate is shown. *p < 0.05; ** p < 0.005; *** p < 0.0005.*

Next, we tested the antiviral activity of a panel of antimetabolites currently used in cancer treatments in wild type or SAMHD1 depleted macrophages. All evaluated drugs inhibited HIV-1 replication, although with different potency (**Table 6**). SAMHD1 expression effectively modified the antiviral activity of all antimetabolites tested. However, and in contrast with previous reports, SAMHD1 degradation either enhanced (cladribine, clofarabine, and nelarabine) or decreased (capecitabine, floxuridine and fluorouracil) the potency of the nucleoside analogues tested (**Figure 19A**). Of note, SAMHD1 degradation dramatically impaired the efficacy of anti-folate inhibitors such as pemetrexed and methotrexate (**Figure 19B**). Calculation of 50% effective concentrations (EC_{50}) of antimetabolites in macrophages expressing SAMHD1 or not showed over 30-fold and 100-fold increases in drugs showing enhanced or diminished potency in SAMHD1-depleted cells, respectively (**Table 6**). The enhanced or decreased efficacy of the compounds tested was not dependent on the nature of the specific nucleotide targeted, i.e., purine or pyrimidine, and was not limited to nucleos(t)ide analogues, as SAMHD1 also affected the efficacy of anti-folate drugs such as pemetrexed and methotrexate (**Table 6**).

A.



B.

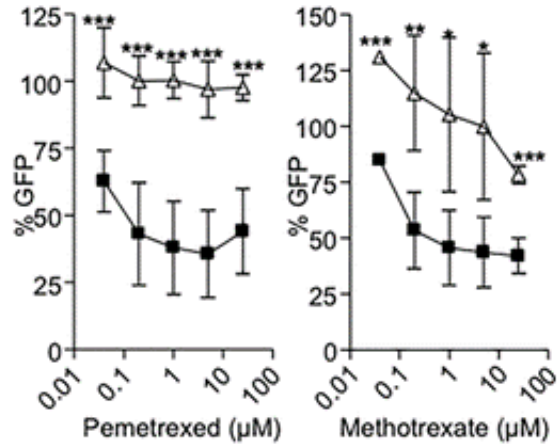


Figure 19. SAMHD1 modifies antiviral and activity of antimetabolites. *Dose response of the nucleoside analogues (**A**) or anti-folate drugs (**B**) currently used as anti-cancer treatments in wild-type (■) or SAMHD1-depleted (Δ) MDMs. Inhibition of HIV infection was measured as the percentage of GFP+ cells relative to the no drug condition. Mean ±SD of at least three independent donors performed in duplicate is shown. *p < 0.05; ** p < 0.005; *** p < 0.0005.*

Table 6. Antiviral activity of antimetabolites tested with or without SAMHD1.

Drugs	Drug Type (Base Targeted)	EC_{50} (μM)		FC	
		SAMHD1 (+)	SAMHD1 (-)	-/+	+/-
AZT	NRTI (dT)	0.006	0.11	18	-
NVP	NNRTI (none)	0.88	0.95	1	1
AraC	Pyrimidine (dC)	3.24	0.11	-	30
Nelarabine	Purine (dG)	13.96	1.83	-	8
Cladribine	Purine (dA)	0.029	0.007	-	4
Clofarabine	Purine (dG)	0.034	0.006	-	6
Gemcitabine	Pyrimidine (dC)	0.02	0.1	6	-
Floxuridine	Pyrimidine (dU)	0.73	20.28	28	-
Fluorouracil	Pyrimidine (dU)	2.40	>25	>10	-
Pemetrexed	Anti-folate	0.25	>25	>100	-
Methotrexate	Anti-folate	0.42	79.24	190	-

EC_{50}; Effective concentration required to block HIV-1 replication by 50%, FC; fold change or ratio of the EC_{50} without SAMHD1 and the EC_{50} with SAMHD1 (-/+), or inversely (+/-).

2.1.2. SAMHD1 is required for antiviral activity of CDK4/6 inhibitors.

Cyclin dependent kinases (CDKs) are a family of proline-directed serine/threonine kinases that were first identified as regulators of cell cycle progression [233]. Mammalian cell cycle progression throughout the G1 phase is sequentially controlled by signalling pathways that regulate the activity of CDK4/6-CyclinD and CDK2-CyclinE/A complexes, which are responsible for modulating the expression, function and stability of many cell-cycle regulatory proteins, including SAMHD1 [234, 235]. In addition, the cellular dNTP pool required for cell division and HIV-1 infection is tightly controlled during the different steps of the cell cycle [183, 236].

SAMHD1 is inactivated in proliferating cells by a mechanism that requires its phosphorylation. SAMHD1 phosphorylation may be directly regulated by CDK1 or CDK2, whose activity is upstream controlled by CDK6. Thus, we evaluated the anti-HIV-1 activity of three highly selective CDK4/6 inhibitors: palbociclib, ribociclib and abemaciclib. As previously shown, the antiviral activity of the CDK4/6 inhibitor palbociclib is dependent on SAMHD1 expression (**Figure 20A**) [183]. Thus, the efficacy of two other specific CDK4/6 inhibitors, ribociclib and abemaciclib, was also evaluated in the presence or absence of SAMHD1. The three agents were tested at the concentration where palbociclib showed the highest efficacy in cell culture (1 μM, **Figure 20A**). As expected, the activity of all three CDK4/6 inhibitors was lost in the absence of SAMHD1, indicating that the efficacy of CDK4/6 inhibitors depends on SAMHD1

expression (**Figure 20B**). Interestingly, similar results were obtained when the multi-kinase inhibitor midostaurin was evaluated (**Figure 21A**), suggesting that activity of multiple types of kinase inhibitors may be influenced by SAMHD1 expression.

To explore the cellular and molecular determinants of SAMHD1 requirement for kinase inhibitor function, SAMHD1 expression and phosphorylation was measured by Western blot. Both palbociclib and midostaurin blocked SAMHD1 phosphorylation, whereas SAMHD1 protein expression was not affected (**Figure 19C and 20B**). In addition, we observed a concomitant dephosphorylation and decreased expression of Rb, a substrate of CDK6, suggesting that palbociclib and midostaurin also affect CDK6-mediated CDK2 phosphorylation of SAMHD1 (**Figure 19C and 20B**).

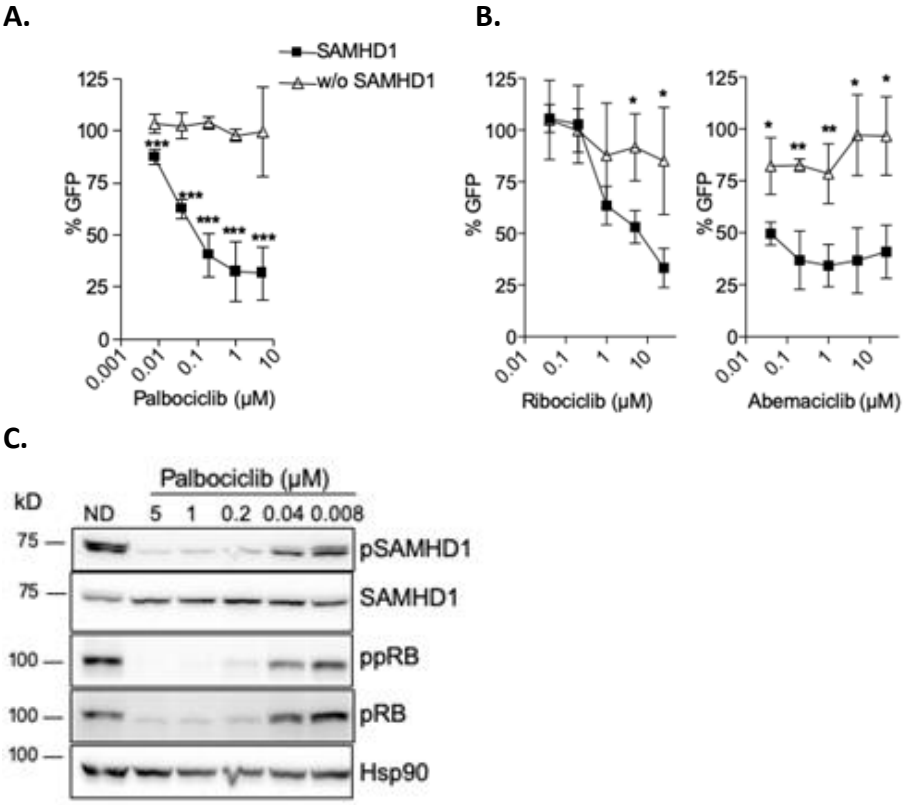


Figure 20. Antiviral efficacy of CDK4/6 inhibitors depends on SAMHD1 expression. ***(A)*** *Dose response of the CDK4/6 inhibitor Palbociclib, in wild-type (■) or SAMHD1-depleted (Δ) MDM. Inhibition of HIV infection was measured as the percentage of GFP+ cells relative to the no drug condition. Mean ±SD of at least ten independent donors performed in duplicate is shown. (**B**) CDK4/6 inhibitors lose antiviral activity in SAMHD1-depleted macrophages. As in **A**, dose response of two other CDK4/6 inhibitors, ribociclib (left panel) and abemaciclib (right panel), in wild-type (■) or SAMHD1-depleted (Δ) MDMs. Mean ±SD of two independent donors performed in duplicate is shown. (**C**) Palbociclib blocks SAMHD1 inactivation by phosphorylation. Western blot analysis of lysates of untreated MDMs (no drug, ND) or macrophages treated with palbociclib at the indicated doses. Membranes were blotted with an anti phospho-SAMHD1 antibody, total SAMHD1, anti phosho-pRB and total pRB. Hsp90 antibody was used as control. A representative donor is shown. *p < 0.05; ** p < 0.005; *** p < 0.0005.*

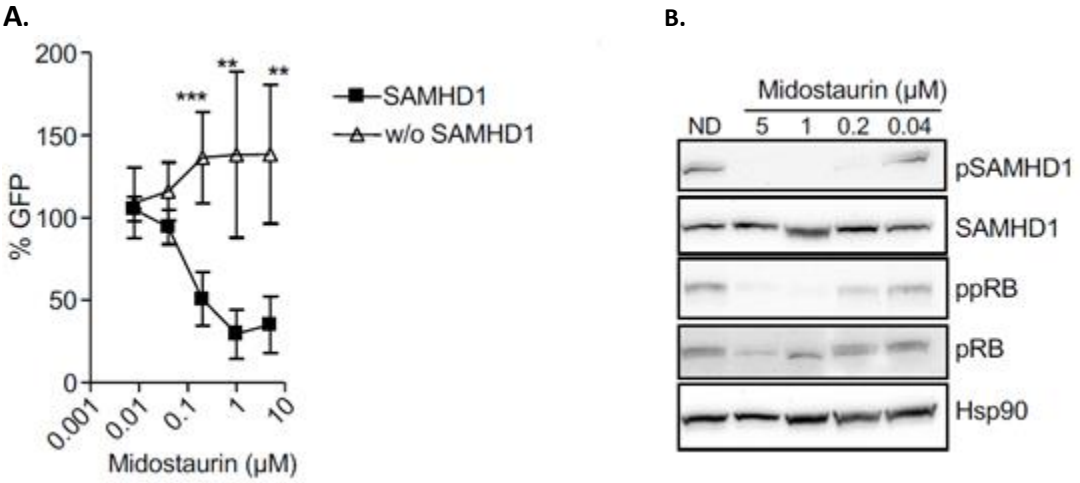


Figure 21. Efficacy of the multi-kinase inhibitor midostaurin depends on SAMHD1 expression. ***(A)*** *Dose response of midostaurin, in wild-type (■) or SAMHD1-depleted (Δ) MDM. Inhibition of HIV infection was measured as the percentage of GFP+ cells relative to the no drug condition. Mean ±SD of at least three independent donors performed in. duplicate is shown. (**B**) Midostaurin blocks SAMHD1 inactivation by phosphorylation. Western blot analysis of lysates of untreated MDM (no drug, ND) or macrophages treated with midostaurin at the indicated doses. Membranes were blotted with an anti phospho- SAMHD1 antibody, total SAMHD1, anti phosho-pRB and total pRB. Hsp90 antibody was. used as control. A representative donor is shown.*

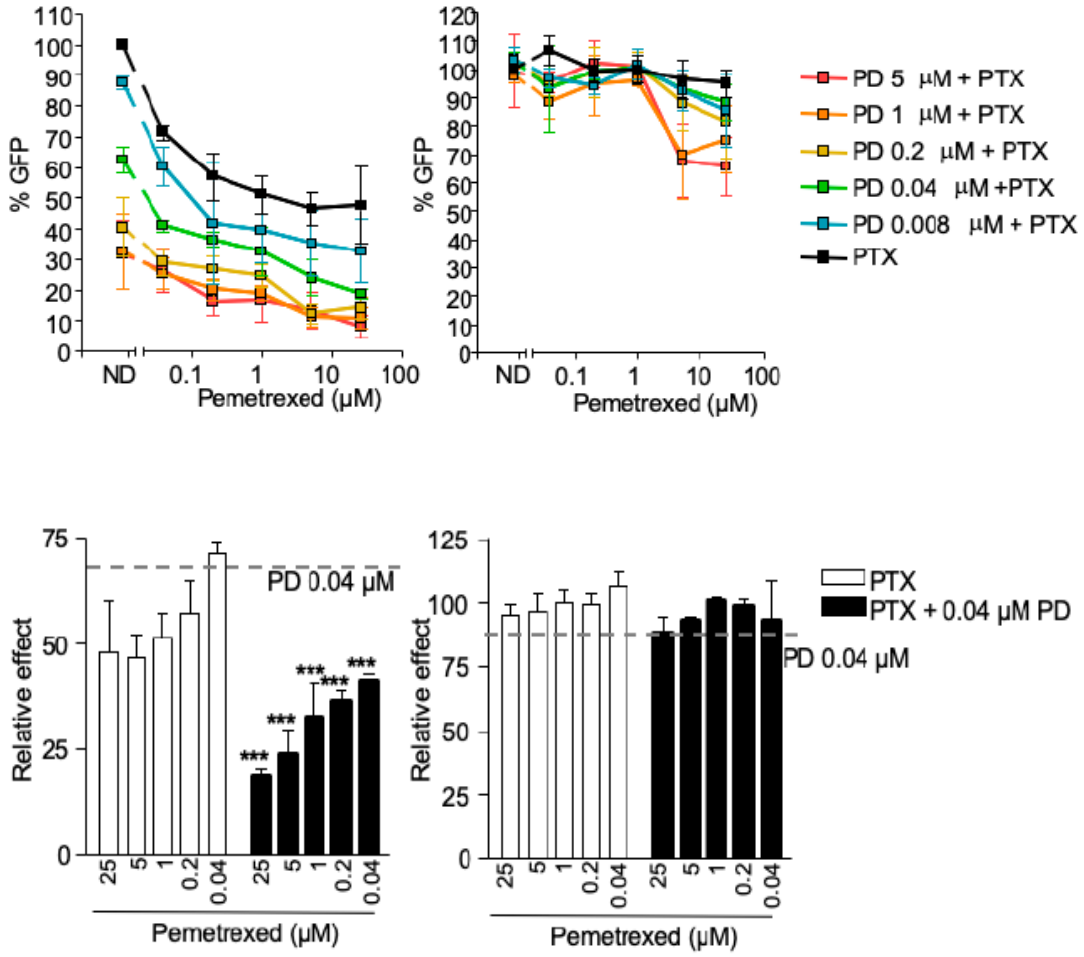
2.1.3. Pharmacological inhibition of CDK4/6 enhances antiviral activity of antimetabolites

CDK4/6 inhibitors activate SAMHD1 function through the inhibition of its phosphorylation. Thus, we evaluated the capacity of palbociclib to modify the activity of antimetabolites. In particular, we selected antimetabolites whose activity is enhanced by SAMHD1 expression, as the agents that could benefit from SAMHD1 activation through dephosphorylation mediated by CDK4/6 inhibitors. Hence, we evaluated the antiviral activity of pemetrexed and fluorouracil alone or in combination with palbociclib in primary macrophages.

Pemetrexed inhibited HIV-1 replication in a dose-dependent manner, although with limited potency (EC50 = 0.1 μM, **Figure 22A**, black line). Combination of pemetrexed with increasing concentrations of palbociclib (EC50 = 0.12 μM) enhanced the antiviral potency of the antimetabolite (**Figure 22A** and **B**, left panels). The calculation of the combination index (CI) indicated strong synergy (CI≤0.041 for palbociclib at 0.04 μM combined with different concentrations of pemetrexed, **Table 7**).

Interestingly, pemetrexed and palbociclib activity, as well as the synergistic effect observed in drug combinations, were lost in the absence of SAMHD1 (**Figure 22A** and **B**, right panels).

A.

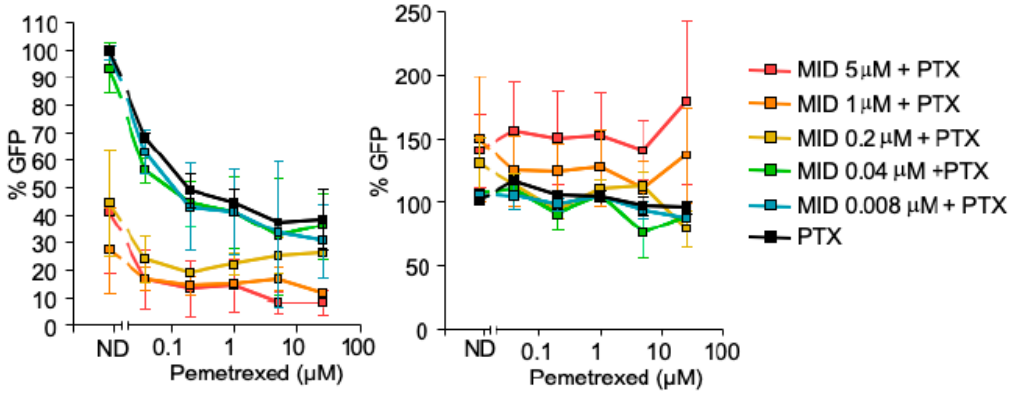


B.

Figure 22. Pharmacological activation of SAMHD1 enhances antiviral activity of antimetabolites. ***(A)*** *Relative effect of the combination of palbociclib-pemetrexed measured as antiviral activity. Inhibition of HIV infection with increasing doses of palbociclib and pemetrexed was measured. Percentage of GFP+ cells relative to the no drug condition is shown in presence (left panel) or absence (right panel) of SAMHD1.* ***(B)*** *As in* ***(A)****, relative effect of pemetrexed alone (white bars) or in combination with a fixed dose of palbociclib 0.04 μM (black bars), in the presence (left panel) or absence (right panel) of SAMHD1.In all experiments, Mean ±SD of at least three independent donors performed in duplicate is shown. PD, palbociclib; PTX, pemetrexed. * p < 0.05; ** p < 0.005; *** p < 0.0005.*

Furthermore, the combination of pemetrexed with the multi-kinase inhibitor midostaurin (EC50 = 0.62 µM) also showed a highly synergistic effect when SAMHD1 was expressed **(Figure 23A and B,** left panels**, Table 7**), an effect that was lost in SAMHD1 depleted cells (**Figure 23A and B,** right panels).

A.



B.

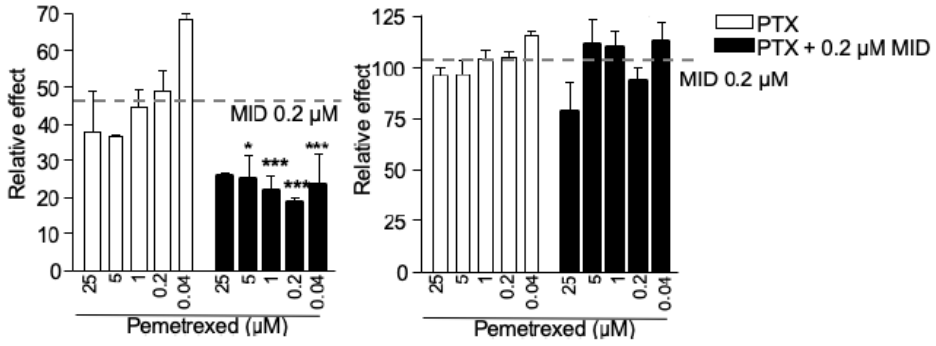


Figure 23. Pharmacological activation of SAMHD1 enhances antiviral activity of antimetabolites. ***(A)*** *Relative effect of the combination of midostaurin-pemetrexed measured as antiviral activity. Inhibition of HIV infection with increasing doses of midostaurin and pemetrexed was measured. Percentage of GFP+ cells relative to the no drug condition is shown in presence (left panel) or absence (right panel) of SAMHD1.* ***(B)*** *As in* ***(A)****, relative effect of pemetrexed (PTX) alone (white bars) or in combination with a fixed dose of midostaurin 0.2 µM (black bars), in the presence (left panel) or absence (right panel) of SAMHD1. In all experiments, Mean ±SD of at least three independent donors performed in duplicate is shown. PTX, pemetrexed; MID, midostaurin. * p < 0.05; ** p < 0.005; *** p < 0.0005.*

On the other hand, combination of the nucleoside analogue fluorouracil or the multikinase inhibitor midostaurin with palbociclib showed more limited effects, i.e., palbociclib partially enhanced the antiviral potency of fluorouracil (**Figure 24A** and **B**) or midostaurin (**Figure 24C** and **D**) in the presence of SAMHD1 (left panels). As expected, no effect was observed when combinatory assays were performed in SAMHD1 depleted cells (right panels). CI calculation indicated synergy at specific concentrations, although CI were 100-fold lower compared to palbociclib-pemetrexed drug interactions (**Table 7**) and antagonist or additive effects were also seen.

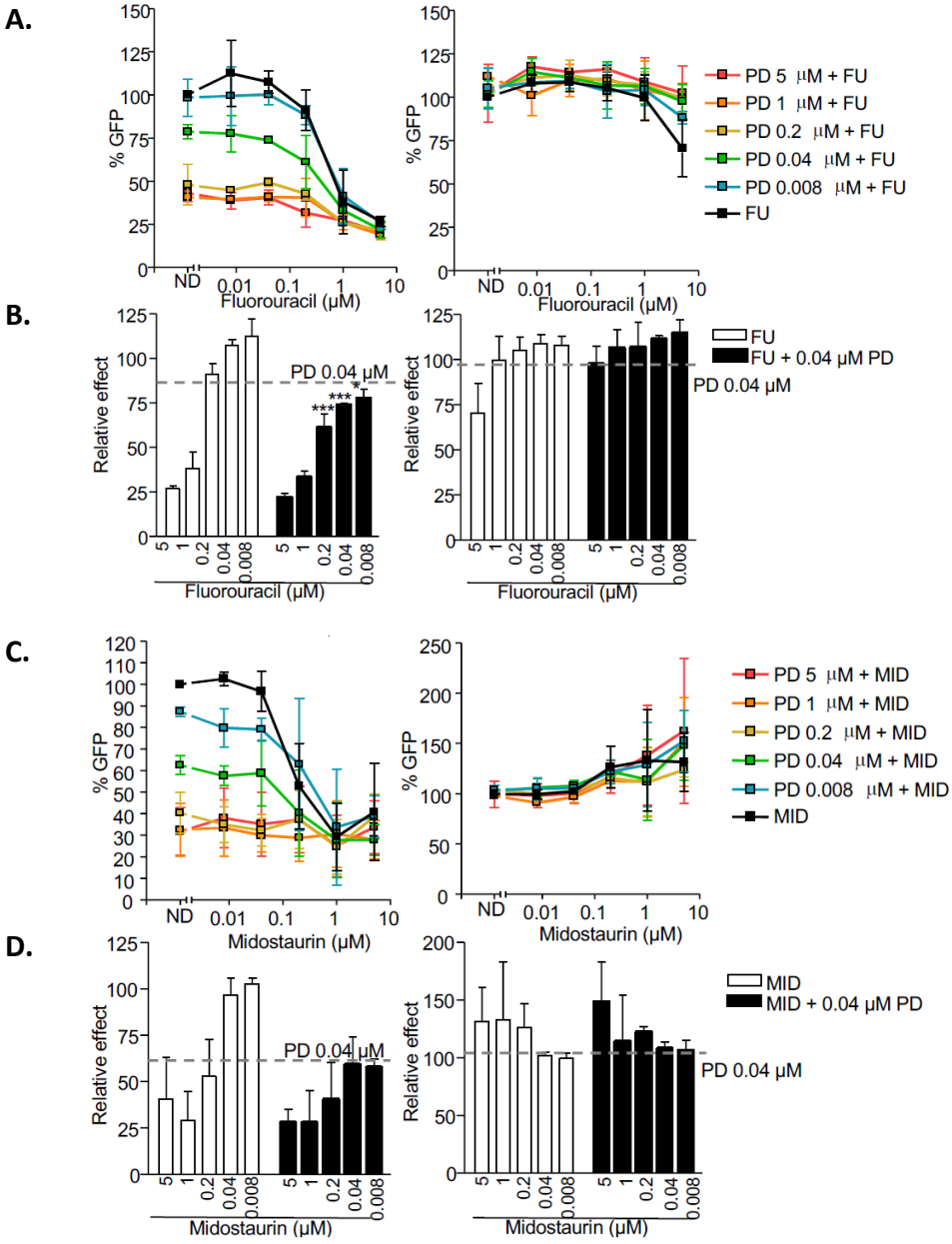


Figure 24. Relative effect of the combination of palbociclib-fluorouracil and palbociblib-midostaurin measured as antiviral activity. ***(A)*** *Inhibition of HIV infection with increasing doses of palbociclib and fluorouracil (FU) was measured. Percentage of GFP+ cells relative to the no drug condition. is shown in presence (left panel) or absence (right panel) of SAMHD1. Mean ±SD of at least three independent donors performed in duplicate is shown. (**B**) As in (**A**) Relative effect of fluorouracil alone (white bars) or in combination with a fixed dose of palbociclib 0.04 μM (black. bars), in the presence (left panel) or absence (right panel) of SAMHD1.Mean ±SD of at least three independent donors performed induplicate is shown. (**C**) Relative effect of the combination of Palbociclib-midostaurin measured as antiviral activity. Inhibition of HIV infection with increasing doses of palbociclib and midostaurin was measured. Percentage of GFP+ cells relative to the no drug condition is shown in presence (left panel) or absence (right panel) of SAMHD1. Mean ±SD of at least 3 independent donors performed in duplicate is shown. (**D**) As in (**C**) Relative effect of midostaurin alone or in combination with a fixed dose of palbociclib 0.04 μM, in the presence or absence (right panel) ofSAMHD1. Mean ±SD of at least 3 independent donors performed in duplicate is shown.*

Table 7. Combination index values for pemetrexed and fluorouracil combinations with palbociclib and midostaurin.

Drug Combination (µM)		Combination Index (CI)	Effect
Pemetrexed	25	0.0049	Synergy
+	5	0.0097	Synergy
Palbociclib 0.04 µM	1	0.0285	Synergy
	0.2	0.0415	Synergy
	0.04	0.0673	Synergy
Pemetrexed	25	0.079	Synergy
+	5	0.069	Synergy
Midostaurin 0.2 µM	1	0.056	Synergy
	0.2	0.045	Synergy
	0.04	0.064	Synergy
Fluorouracil	5	1.871	Antagonism
+	1	0.572	Synergy
Palbociclib 0.04 µM	0.2	0.658	Synergy
	0.04	1.818	Antagonism
	0.008	2.967	Antagonism
Midostaurin	5	2.074	Antagonism
+	1	0.427	Synergy
Palbociclib 0.04 µM	0.2	0.223	Synergy
	0.04	0.419	Synergy
	0.008	0.324	Synergy

CI values were calculated using the mean values of three different experiments. Values were calculated using CompuSyn software. CI < 1, synergy; CI > 1, antagonism; CI = 1, additive.

Overall, these results suggest that pharmacological activation of SAMHD1 can significantly enhance the efficacy of antimetabolites, through a mechanism that is dependent on SAMHD1 expression and regulation.

2.2. Cytotoxic efficacy modification of anticancer agents by SAMHD1

The finding that pharmacological modulation of SAMHD1 by CDK inhibitors is able to enhance the antiviral activity of a wide range of chemotherapeutic agents in MDMs, reveals the potential of this therapeutic approach for the development of novel strategies in the treatment of cancer.

2.2.1. Cytotoxic efficacy of antimetabolites is enhanced by CDK4/6 inhibitors.

Anticancer drugs are specifically designed to inhibit cell growth, thus we evaluated cytotoxic efficacy of the antimetabolites pemetrexed and fluorouracil in combination with the CDK4/6 inhibitor palbociblib in the TZM-bl cell line and in two distinct breast cancer cell lines, MDA-MB-468 and T47D. First, we evaluated CC_{50} of the different compounds alone to determine the most appropriate concentration for the combinatory assay (**Table 8**, **Figure 25**). As expected, all drugs tested resulted in decreased cell metabolic activity in all cell lines, reflecting the number of viable cells under defined conditions.

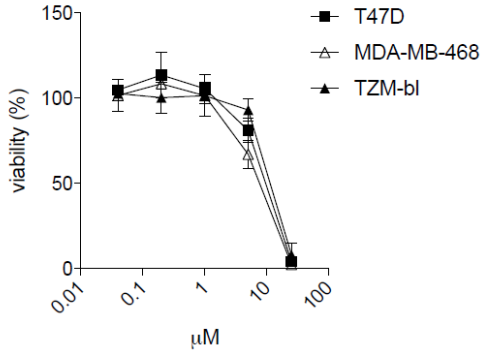


Figure 25. **Cytotoxic activity of palbociclib in cell lines.** *Cell viability was measured after palbociclib treatment, observing a clear dose-response. Mean ±SD of at three independent experiments performed in triplicate is shown. Cytotoxic effect of the drugs was tested by MTT assay.*

Table 8. Cytotoxic activity of drugs evaluated in the different cell lines.

Drug	CC_{50} (µM)		
	TZM-bl	MDA-MB-468	T47D
Pemetrexed	0.386	7.507	18.251
Fluorouracil	24.617	114.785	227.419
Palbociclib	11.693	7.651	9.586

CC_{50}: Cytotoxic concentration required to block cell replication by 50%

The combination of pemetrexed with palbociclib enhanced the cytotoxicity of the antimetabolite in all cell lines tested (**Figure 26A-C**). Importantly, the calculation of the combination index indicated a synergistic effect in all cases, with the cytotoxic evaluation being comparable to the results obtained when antiviral efficacy was measured (**Table 9**). The combination of fluorouracil with palbociclib enhanced fluorouracil potency in TZM-bl and T47D cells but not in MDA-MB-468 cell line (**Figure 26A-C**, **Table 9**).

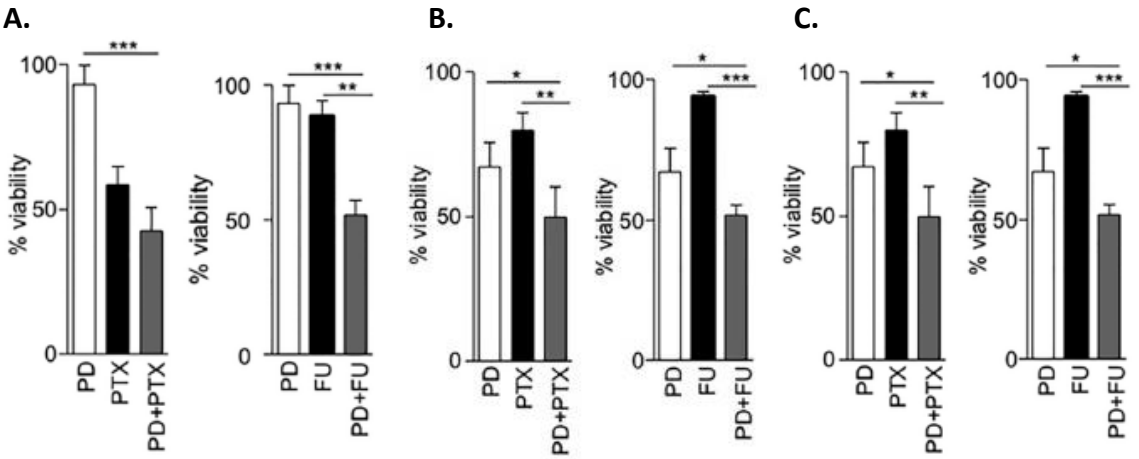


Figure 26. Pharmacological activation of SAMHD1 enhances cytotoxicity of antimetabolites. *Effect on cell viability of palbociclib–pemetrexed combination in TZM-bl (**A**), T47D (**B**) and MDA-MB-468 (**C**) cell lines, respectively. Left panels, cytotoxic activity of palbociclib alone (5 µM, white bars), pemetrexed alone (black bars at 0.2, 1 or 0.04 µM for TZM-bl, T47D and MDA-MB-468 respectively) or the combination of both drugs at the same concentration (grey bars). Right panels, cytotoxic activity of palbociclib alone (5 µM, white bars), fluorouracil alone (5 µM, black bars) or the combination of both drugs at the same concentration (grey bars). Drug concentrations were chosen depending calculated CC50 under specific experimental conditions. * p < 0.05; ** p < 0.005; *** p < 0.0005. PD, palbociclib; PTX, pemetrexed; FU, 5-fluorouracil.*

Table 9. Combination index values for pemetrexed and fluorouracil combinations with palbociclib in cancer cells.

Cell Type	Drug Combination		Combination index (CI)	Effect
TZM-bl	Pemetrexed	1	0.806	Synergy
	+	0.2	0.689	Synergy
	Palbociclib 5 µM	0.04	0.710	Synergy
		0.008	0.720	Synergy
	Fluorouracil	5	0.726	Synergy
	+	1	0.766	Synergy
	Palbociclib 5 µM	0.2	0.764	Synergy
		0.04	0.735	Synergy
T47D	Pemetrexed	5	0.783	Synergy
	+	1	0.707	Synergy
	Palbociclib 5 µM	0.2	0.779	Synergy
		0.04	0.874	Synergy
	Fluorouracil	25	0.848	Synergy
	+	5	0.804	Synergy
	Palbociclib 5 µM	1	0.966	Additive
		0.2	1.010	Additive
MDA-MB-468	Pemetrexed	1	0.886	Synergy
	+	0.2	0.886	Synergy
	Palbociclib 5 µM	0.04	0.921	Synergy
		0.008	1.010	Additive
	Fluorouracil	25	2.844	Antagonism
	+	5	1.745	Antagonism
	Palbociclib 5 µM	1	1.356	Antagonism
		0.2	1.125	Antagonism

CI values were calculated using the mean values of three different experiments. Values were calculated using CompuSyn software. CI < 1, synergy; CI > 1, antagonism; CI = 1, additive.

We then characterized by western blot the expression signature and phosphorylation status of SAMHD1 and pRb, a critical protein during cell cycle, of the cancer cell lines tested. Interestingly, in MDA-MB-468 cells, although the expression of SAMHD1 was similar to other lines, Rb and pRb were not detected, either at the mRNA or protein level, (**Figure27**), demonstrating the importance of cell cycle proteins which putatively may affect SAMHD1 function in determining palbociclib–antimetabolite drug combination efficacy.

Figure 27. Protein expression in the different cell lines used. *Western blot showing expression and phosphorylation of pRB and SAMHD1 in T47D, MDA-MB-468 and TZMbl cell lines. Membranes were blotted with an anti phospho-SAMHD1 antibody, total SAMHD1, anti phosho-pRB and total pRB. Hsp90 antibody was used as control. A representative experiment is shown.*

2.2.2. Alternative pathways of dNTP metabolism control are responsible for drug synergy.

To further explore the mechanism underlying the synergistic effect observed when combining antimetabolites with the CDK4/6 inhibitor palbociclib, protein expression in primary macrophages treated with pemetrexed, fluorouracil and palbociclib, alone or in combination was evaluated (**Figure 28**). As expected, palbociclib alone inhibited phosphorylation of pRb and SAMHD1, therefore activating its dNTP triphosphohydrolase function and subsequently reducing the intracellular dNTP pool. Interestingly, pemetrexed and fluorouracil treatment resulted in different effects, i.e., while fluorouracil acts similarly to palbociclib, pemetrexed did not decrease the phosphorylation of pRb and SAMHD1. Although pemetrexed activity is dependent on SAMHD1, its mechanism of action does not directly affect SAMHD1 phosphorylation, providing evidence for the stronger synergy observed in the pemetrexed–palbociclib drug combination compared to fluorouracil–palbociblib.

Thus, antifolates such as pemetrexed inhibit the dNTP pool by a mechanism not directly affecting SAMHD1 phosphorylation and effectively synergized with palbociclib, which induces SAMHD1 activation. On the other hand, when two compounds directly affecting SAMHD1 phosphorylation (i.e., fluorouracil and palbociclib) are combined, the synergic effect is less potent (**Figure 29**).

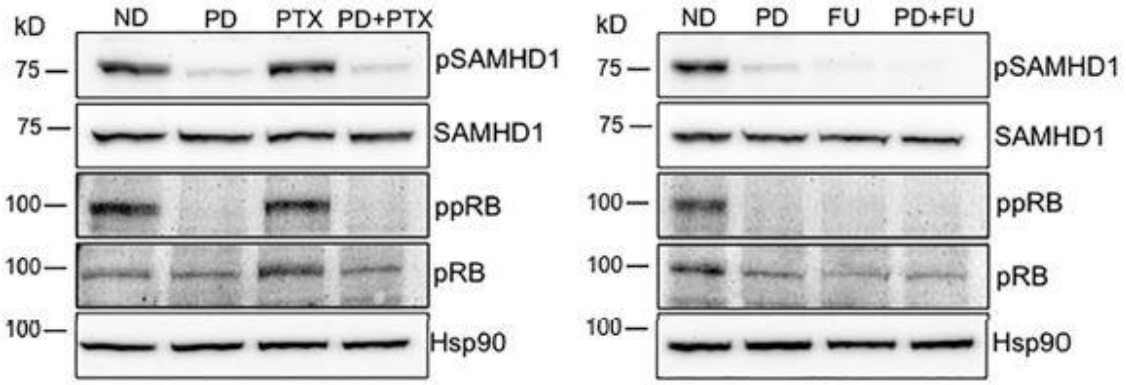


Figure 28. **Regulation of dNTP pool is responsible for drug synergy.** *Protein expression in MDMs treated with palbociclib (PD) at 1 µM, pemetrexed (PTX) and fluorouracil (FU), both at 5µM and the corresponding drug combinations PD+PTX and PD+FU. Hsp90 was used as a loading control. A representative blot is shown. Consequently, the antiviral and cytotoxic efficacy of antimetabolites is significantly enhanced when used in combination in vitro.*

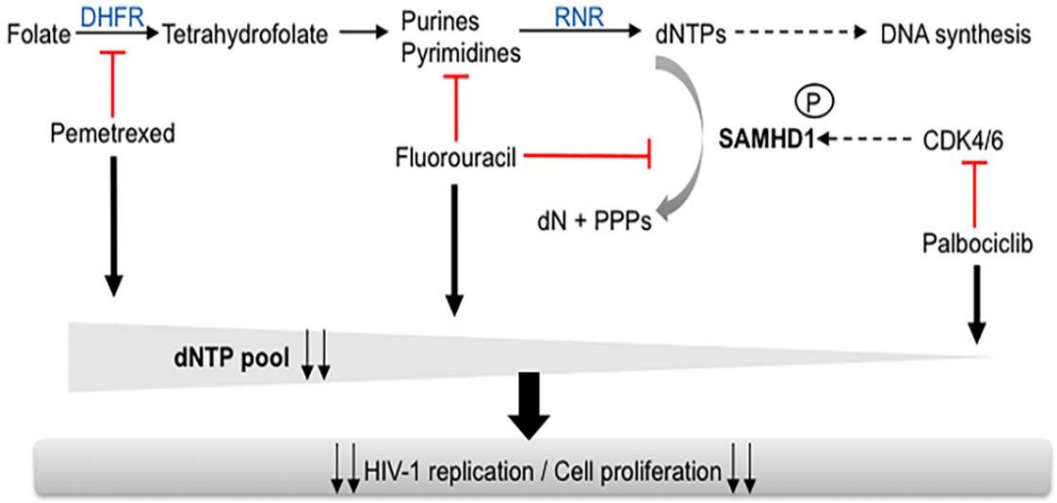


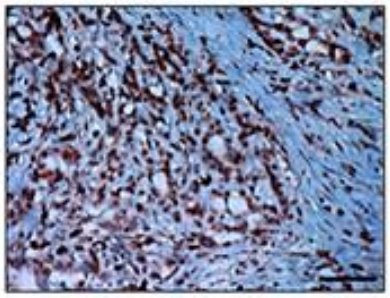
Figure 29. Proposed model of drug interactions. *Antimetabolites affecting dNTP synthesis such as pemetrexed inhibit dNTP pool by a mechanism not directly affecting SAMHD1 activation and thus synergy with anticancer drugs affecting SAMHD1 phosphorylation as palbociclib is higher compared to compounds also targeting SAMHD1 function (i.e., fluorouracil) or exclusively affecting SAMHD1 (i.e., midostaurin).*

2.3. SAMHD1 is expressed in different tumor tissues

To explore the potential value of modulating SAMHD1 function in cancer patients, we evaluated SAMHD1 expression in different tumors by immunohistochemistry (IHC) in paraffin-embedded tissues. SAMHD1 was clearly detected in at least two cancer tissue types susceptible of being treated with antimetabolites, pancreatic adenocarcinoma and non-small lung cell carcinoma (**Figure 30A** and **B**). In both cases, SAMHD1 was significantly expressed in a high percentage of malignant cells.

In addition, IHC data of 17 different types of human tumors including 202 different samples from human protein atlas were also analyzed (www.proteinatlas.org). Although SAMHD1 was expressed in all types of tumors, the degree of expression was significantly variable, ranging from undetectable levels to high protein expression levels. Overall, 70% of all tumors expressed SAMHD1 to a certain extent, whereas its expression could not be detected in 30% of cases (**Figure 28C**). These results demonstrate that SAMHD1 is expressed in patient tumor samples but also suggest that modulation of SAMHD1 function might be feasible at least in a subgroup of cancer types.

A.



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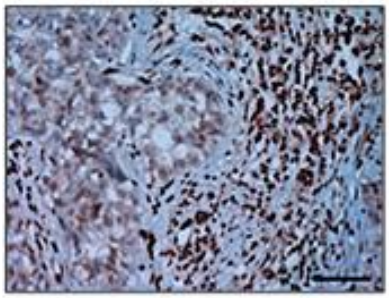

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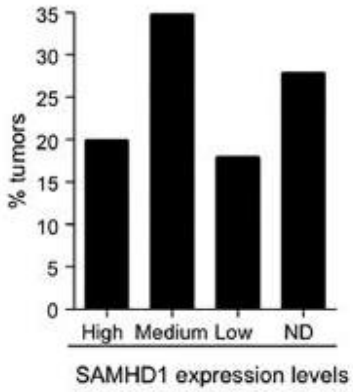

Figure 30. Expression of SAMHD1 protein in tumor samples from cancer patients. *IHC staining of SAMHD1 in pancreas (**A**) and lung (**B**) tumor samples. Morphology of tumor cells is shown by routine hematoxylin stain of paraffin embedded tumor sections. SAMHD1 is stained in brown. Original magnification ×200. Scale bar, 20 µm. (**C**) Percentage of tumors expressing SAMHD1, depending on its relative expression in IHC as classified in Human Proteome Atlas (www.proteinatlas.org, IHC data of SAMHD1 expression from 17 different tumor types were retrieved and classified according to protein expression levels. ND, not detected.*

In summary, here, demonstrate that SAMHD1 can enhance or decrease the efficacy of various classes of anticancer drugs, including nucleotide analogues, but also anti-folate drugs and CDK inhibitors. Importantly, we show that selective CDK4/6 inhibitors are pharmacological activators of SAMHD1 that act by inhibiting its inactivation by phosphorylation. Moreover, combinations of a CDK4/6 inhibitor with nucleoside or folate antimetabolites potently enhanced drug efficacy, resulting in highly synergic drug combinations (CI<0.04). In addition, mechanistic analyses reveal that cell cycle-controlled modulation of SAMHD1 function is the central process explaining changes in anticancer drug efficacy, therefore providing functional proof of the potential of CDK4/6 inhibitors as a new class of adjuvants to boost chemotherapeutic regimens.

The evaluation of SAMHD1 expression in cancer tissues allowed for the identification of cancer types that would benefit from the pharmacological modulation of SAMHD1 function. In conclusion, these results indicate that the modulation of SAMHD1 function may represent a promising strategy for the improvement of current antimetabolite-based treatments.

DISCUSSION AND PERSPECTIVES

The extensive and thorough study of genetic and molecular factors associated with different intracellular processes has provided unique insights in the understanding of the physio-pathological mechanisms that mediate diverse human diseases, such as autoinflammation, viral infections and cancer. Collectively, these studies have significantly advanced our understanding of the immune system and the mechanisms underlying a broad range of human diseases, including HIV infection and cell transformation processes leading to cancer. This knowledge has also guided research into the generation of cutting-edge strategies for the development of novel therapeutical approaches. The present work is framed within this conjecture and aimed to participate in the description of cellular factors with potential impact for a better understanding of human diseases such as HIV infection and cancer, that might lead to the proposal of novel treatments.

People leaving with HIV have an increased risk of developing different types of cancer. In addition, HIV infection and cancer exploits similar cellular processes and molecular pathways regarding to pathogenic mechanisms and immune system weakening. Here, we focus on the detailed study of two of these common features that could be key for the development of novel therapeutic approaches against HIV and cancer.

The main role of the human immune system is to eliminate cells presenting foreign antigens and abnormal patterns. However, depending on the circumstances, the immune system alone cannot completely eliminate the anomalies, leading to the establishment of pathologies. Innate immunity is the first line of defence common to a wide range of pathologies, from infection to cancer. Albeit innate immune system has been considered as unspecific, in recent years, it has become clearer that the modulation of innate immune system might be a relevant tool for the treatment of human diseases such as HIV and cancer.

HIV infection and cancer may be considered as unsolved chronic diseases and there is an urgent need to identify novel cellular targets for the development of alternative therapeutic interventions. Here, we applied the knowledge generated in the context of HIV-1 latency and HIV treatment to explore the role of cellular factors as targets for the development of novel strategies to better understand and treat HIV infection and cancer. This thesis focuses on the role of two distinct factors that might change the

course of HIV infection and cancer development, the Fc-gamma receptor CD32 and the dNTP triphosphohydrolase SAMHD1, both evaluating disease onset and progression in the context of HIV infection but also in response to current anticancer therapies.

Presence of latent HIV reservoirs forms the major obstacle to an HIV cure. Hence, the molecular identification of these latent cells is the most important challenge for achieving a definitive HIV cure [243-245]. The best characterized and the most likely mechanism for HIV persistence is the generation and maintenance of a “silent” reservoir of proviruses mainly in resting memory CD4+T cells. Latently HIV-1-infected CD4+ T lymphocytes are *a priori* indistinguishable from uninfected lymphocytes and persist even during effective ART [243, 249, 250]. Several unsuccessful attempts for the identification of molecular markers for such latently infected cells have been reported [251-260].

It has been proposed that expression of immune checkpoints (PD-1, CTLA-4, LAG-3, TIGIT, TIM-3, CD160 and CD244), typically upregulated in cancer processes, are positively associated with CD4+ T cells harbouring integrated HIV DNA and enriched for HIV infection under ART with a higher tendency to viral transcription [77, 251-255]. Additional surface proteins such as CD2, CD30 or CD20 have been proposed as putative latency markers, though with modest evidence of viral DNA/RNA enrichment [79, 80, 82]. Immune checkpoints inhibitors are already currently used to treat malignancies and had been proposed as reactivating agents of HIV expression from latency [256, 257], although with conflicting results [258-260]. However, although some promising possibilities had been proposed, no definitive HIV latency marker has been discovered so far. While some of the proposed biomarkers could point to some cell subsets enriched with HIV DNA or RNA, all of them failed to simultaneously identify transcriptionally silent infected cells and cells capable to yield productive infection after stimulation.

At first sight, one of the most encouraging proposed markers of the HIV reservoir has been the immunoglobulin receptor CD32, reported by Descours et al.[83] In this study, an enrichment (~1000-fold) in HIV DNA was observed in CD4+ T cells with CD32 expression as compared to CD32 negative cells, in contrast to all previously suggested markers [83]. Besides, they also demonstrated an enrichment for replication-competent proviruses in these cells. If confirmed, these findings would represent a milestone in the

efforts to develop a cure for HIV infection. However, subsequent reports have questioned the reproducibility of these findings.

In the first chapter of this work, we aim to characterize the pattern of expression of CD32 using both, uninfected and *in vitro* and *ex vivo* models of acute and latent HIV infection. When evaluating stimulated uninfected PBMCs, we found that CD32 expression is strongly associated with CD4+ T cells that co-express the activation markers HLA-DR and/or CD69, which are in line with previous and subsequent reports [94, 96, 263-265]. Thus, exogenous activation of purified CD4+ T cells with different stimulus induced CD32 cell surface expression correlating with cell proliferation marker (Ki67+), concluding that CD32 expression is a marker of activation in a subset of CD3+CD4+ T cells, as recently proposed [231].

Activation of CD4+ T cells is associated with HIV-1 pathogenesis and the establishment of an HIV-1 reservoir. Indeed, the HIV-1 reservoir is thought to form cells that are infected while activated before returning to a resting state [261]. According to this, HIV+ cells expressing HLA-DR could not be considered as latently infected, since they are transcriptionally active. In this regard, Grau-Expósito et al. first reported the association between CD32 and HLA-DR overexpression after *ex vivo* HIV-1 infection of unstimulated PBMCs [262]. Our results also show that CD32 expression is enhanced by HIV infection similar to HLA-DR upregulation during T-cell activation, thus CD32 expression may identify a subset of activated CD4+ T cells that are susceptible to HIV infection. This finding further indicates that CD32 is a consequence of T-cell activation induced either by exogenous stimuli or by HIV-1 infection. Recently this year, Adams et al., showed increased expression of HLA-DR, CD38 and CD69 in CD4+CD32+ memory T cells from the infected tissues of combined ART-treated humanized mice, similar to our results from peripheral blood lymphocytes [272]. They reported that CD32+CD4+ memory T cells have a high activation/exhaustion profile during suppressed viremia [272]. In line with our results, we shown that CD32+CD4+ T cells co-expressed the activation markers HLA-DR and CD69. Our data suggest that establishment of HIV-1 latency may be the consequence of infection in CD4+T cells within a narrow window of time after activation. Thus, CD32 expression may signal a transition state to or from a fully susceptible phenotype.

CD32 may identify a highly activated/exhausted subset of memory CD4+ T cells that might subsequently favour an HIV-1 enrichment in these cells. However, we found no significant differences between the ratio of infected (GFP+) cells in CD32+ compared with CD32− cells, indicating that CD32 is not a preferential marker for infection, even though the majority of CD32+ cells also co-express the activation marker HLA-DR. Using a model of HIV latency in CD4+ resting cells, we also showed that infection induces CD32 expression. Indeed, proviral DNA in *in vitro* infected resting cells was preferentially found in the CD32− cell compartment. Evaluation of cells from HIV+ individuals showed similar results, further indicating the link between CD32, HIV infection and T cell activation. Accordingly, other studies in peripheral blood of HIV-1-infected patients are in agreement with our findings [94-96]. Controversially, Darcis, et al. demonstrate a prominent enrichment of proviral DNA in CD32 expressing cells after multiple rounds of CD4+ T cells purification [97]. Notably, the putative contribution of CD32+ CD4+ T cells to the HIV reservoirs seems to be highly variable from one HIV-infected individual to the other, both in peripheral blood and in tissues [80, 97, 264- 266, 270].

Overall, our data challenge the robustness of CD32 as a marker of an HIV-1 reservoir. We found that in the majority (6 out of 10) of HIV+ individuals, the absolute contribution to the CD4+ T cell HIV-1 reservoir was higher in CD32− CD4+ T cells. Descours, et al. raw data showed the same results, in which the absolute contribution of HIV-proviral DNA copies by CD32+ cells was higher in only 5 of 9 HIV+ individuals, and in one, the contribution was comparable between CD32− and CD32+ cells. Similarly, other studies also observed limited or no enrichment for total HIV DNA or for replication competent proviruses in CD32+ cells [95, 96, 263, 264].

Furthermore, our results indicate that there are no significant differences between replication competence of viruses emerging from CD32− and CD32+ CD4 T cells. While the TZM-bl assay used in our study may be overestimating replication-competent HIV-1, we compared the viral outgrowth of cultures with an equal cell number for CD32− and CD32+ cells, allowing for head-to-head comparisons between both cell types. Taken together, our results do not support the existence of a distinct population of latent provirus in CD32+ and CD32− cells; both cell types harboured similar amounts of integrated provirus with similar replication competence.

The correlation between cell activation and CD32 expression may suggest that CD32+ CD4+ T cells have a history of activation consistent with the current understanding of how the reservoir develops and is maintained. Specifically, it has been described that HIV-1-infected resting memory CD4+ T lymphocytes mirror a post-activation state, in which infection and subsequent return to a lower activation level occurred [263]. The hypothesis that CD32 cell surface expression would allow for the selective recognition of an HIV-1 reservoir contradicts the paradigm of the "undistinguishable phenotype of latently infected cells". Considering that Descours, et al. suggested that HIV-1 infection leads to CD32 expression and likely 103 other genes [83], this data could be interpreted as indicative that latently infected cells would no longer be resting cells. Our data suggest that CD32 expression represents a marker of activation in a subset of CD4+ T cells, rather than a marker of the HIV-1 reservoir. However, the observed association between immune activation and viral persistence suggests that these two phenomena may be reciprocally connected; a putative role of CD32 in such a scenario cannot be ruled out, as it has been suggested that HIV can establish latent infection in activated CD4+ T cells [266, 267, 268]. The existence of latently infected CD4+ cells that are activated, and therefore relatively short-lived, could suggest continuous replenishment of this component of the reservoir by cellular proliferation [51, 269]. Nonetheless, the need to redefine the mechanisms of establishment and persistence of the HIV reservoir remains a pending issue, and a better characterization and immune phenotyping of the CD32+ cell subsets is important to clearly determine the exact contribution of CD32-expressing cells to the replication-competent latent reservoir.

The profound understanding of the complex mechanisms and molecular pathways involved in the induction and maintenance of HIV latency are critical to develop an effective therapeutical strategy. In human T cells, infection with HIV-1 causes cell-cycle arrest or delay in the G2 phase of the cell cycle. Viral accessory proteins have been shown to alter the cell cycle by inhibiting the activation of CDK1, a kinase controlling the G2/M checkpoint, to prevent or delay entry of infected cells into mitosis [273-276]. Further demonstrating the tight link between cell cycle control and the establishment of HIV-1 infection and latency. Hence, cell cycle modulation and related host factors might also play a crucial role in the establishment and maintenance of HIV-1 latency.

The host factor SAMHD1 restricts HIV-1 replication in nondividing cells by degrading intracellular dNTPs and is highly expressed in resting CD4+ T lymphocytes, the subset of cells considered to preferentially host the HIV reservoir. SAMHD1 function is tightly linked to cell cycle mainly due to its regulation through CDK-mediated phosphorylation, which controls SAMHD1 activation [173-176]. According to the classical model of cell cycle control, CDK6, together with CDK4, regulate cell cycle entry from G0 to G1 and the subsequent activation of CDK1/2 during G1-S transition. Phosphorylation of SAMHD1 inactivates its triphophohydrolase activity, a process that is mediated either by CDK1 or CDK2, leading to an increase of the dNTP pool and the deactivation of the viral restriction activity [173-176]. Conversely, in non-cycling cells such as resting CD4+T lymphocytes, CDK1/2 remain inactive during G0 phase and thus, SAMHD1 predominates in an active dephosphorylated state leading to reduced dNTP levels and active HIV restriction [172, 177, 178].

We show that selective CDK4/6 inhibitors (palbociclib, ribociclib, abemaciclib) but also multi-kinase inhibitors as midostaurin, present anti-HIV activity in MDM only in the presence of SAMHD1, demonstrating that activation through dephosphorylation of the enzyme boosts its triphosphohydrolase activity enhancing HIV restriction [183]. Accordingly, other tyrosine kinase inhibitors such as dasatinib and ponatinib, used for treating chronic myeloid leukemia, has been described to block HIV-1 replication and the potential expansion of the latent reservoir by interfering with SAMHD1 phosphorylation [289, 290]. In consequence, since the establishment of HIV latency occurs during the first steps of infection, modulation of SAMHD1 function through CDK inhibitors may impact not only acute HIV infection but also it might influence the establishment and reactivation of the HIV reservoir. In this line, it has been reported the correlation between SAMHD1 binding to the HIV-1 LTR and SAMHD1-mediated suppression of viral gene expression and reactivation of HIV-1 latency, suggesting that SAMHD1 is among the host proteins involved in the transcriptional regulation of proviral DNA [278, 288]. Therefore, using CDKs inhibitors to modulate SAMHD1 activity, alone or in combination with other first-line drugs, harbours the potential for developing novel treatments to target the HIV reservoir.

In addition to the differential activity of CDK4/6 inhibitors in the presence or absence of SAMHD1, it has been reported that SAMHD1 could modify the efficacy of several nucleoside analogues, either used as antiretrovirals [201-205] or as chemotherapeutic drugs [206-208]. Nucleotide analogues are a large class of drugs that prematurely terminates the DNA synthesis generally through the binding of viral and/or cellular polymerases. Therefore, nucleotide analogues have been widely used as antiviral and anticancer agents, to treat rheumatologic diseases and even bacterial infections, and are the current gold-standard for multiple viral infections and the treatment of choice for many malignancies. Indeed, the first FDA approved anti-HIV medication, zidovudine, was initially developed as an anti-cancer medicine in the late 1960s [238]. Besides, it has been shown that the NRTI, abacavir, is able to induce antiproliferative activity and trigger senescence in prostate cancer cells [239]. Other nucleoside-based antiviral agents such as cidofovir and ganciclovir, have been widely investigated for their ability to induce cell death in rapidly dividing cancer cells [240, 241]. Further, the NNRTI, efavirenz, has been demonstrated to have profound antiproliferative activity against pancreatic cancer as well as anaplastic thyroid cancer [242].

Combination therapies still constitutes the current paradigm to achieve systemic disease control in HIV infection and clinical oncology [282]. Taken into account SAMHD1 role, the pharmacological modulation of its activity may represent a new approach for improving efficacy of current therapeutic options, used to treat HIV infection and cancer. Hence, in the second chapter of this thesis, we aim at evaluating the potential of SAMHD1 as a modulator of antimetabolite therapy.

By using an in-house highly sensitive HIV-based assay, we evaluated SAMHD1 capacity to impact on drug efficacy. Our assay was able to identify distinct types of changes in drug efficacy that depend on SAMHD1 function. Based on our results and previous data showing that triphosphorylated nucleoside analogues such as Ara-C can be hydrolysed by SAMHD1 [133-135], we identify compounds whose activity is enhanced in the absence of SAMHD1, i. e., enzyme substrates. On the contrary, and as previously demonstrated for NRTI [202-204], compounds that gain activity in the presence of SAMHD1 would behave as competitors of the intracellular dNTP pool, which is lower when SAMHD1 is active. Thus, SAMHD1 is able to enhance efficacy of a wide range of

antimetabolites, not only of nucleoside analogues but also of other drugs modifying nucleoside metabolism. Accordingly, anti-folates used in cancer therapy such as pemetrexed or methotrexate [277, 286] showed higher anti-HIV activity when SAMHD1 effectively limits the dNTP pool, which brings further evidence supporting our hypothesis.

Although our results seem to indicate that purine nucleoside analogues may be more prone to gain activity in SAMHD1-depleted cells, SAMHD1 effect on drug efficacy did not depend on the chemical structure of the specific base targeted. Interestingly, here we report the enhancement of nelarabine activity in SAMHD1 depleted cells. In line with our results, it has been recently shown by Rothenburger, et al. that there is an inverse correlation between nelarabine sensitivity and the expression of SAMHD1 in T-cell acute lymphoblastic leukaemia (T-ALL). In this study, lower expression of SAMHD1 in T-ALL cells increased the cytotoxic activity of nelarabine compared to B-cell acute lymphoblastic leukaemia (B-ALL), where SAMHD1 levels are higher [281]. Notably, these findings suggest that leukaemia patients may benefit from SAMHD1 inhibition in combination with nelarabine therapy.

Additionally, our detection assay of antimetabolite sensitivity, has allowed us to reveal a distinct dependency on SAMHD1 expression of two highly similar drugs, structurally and metabolically, as gemcitabine and cytarabine, but showing an inverse correlation with SAMHD1 expression. Hollenbaugh et al., were the first to identify that gemcitabine was not a substrate of SAMHD1 [210] and subsequent studies done by Rudd et al., provided additional probe on the role of gemcitabine as a SAMHD1 functional inhibitor [285]. Our results obtained from the antiviral assays support and provide further evidence for these findings.

Overall, we have developed a simple and highly sensitive screening approach based on anti-HIV-1 activity in primary macrophages. First, SAMHD1 expression is easily modulated through HIV-2 Vpx but also, HIV-1 reverse transcription is a process highly sensitive to dNTP pool sizes and can be easily monitored. Second, cell cycle initiation and progression are not deregulated and by using primary cells, we may also be considering inter-individual differences. Additionally, MDMs shows an intrinsic

resistance to the cytotoxic effects of chemotherapeutic agents. Thus, HIV-1 infection of MDM provides an excellent model to test the activity of antimetabolite drug efficacy.

Our results might also represent the first step for the proposal of novel treatment strategies directed to the modulation of SAMHD1 function. Thus, we tested the capacity of CDK4/6 inhibitors to activate SAMHD1 impeding its phosphorylation and therefore boost antimetabolite-based anticancer therapies, especially for drugs whose activity is enhanced by SAMHD1. Indeed, cytotoxicity data obtained in different cell lines confirmed the results from the antiviral-based screening, showing a strong synergy when combining pemetrexed with kinase inhibitors. Additionally, further investigation of antiretroviral agents is needed to assess the potential of SAMHD1 activity modulation to improve antiviral therapy.

Importantly, nowadays CDK4/6 inhibitors in combination with endocrine therapies is the standard treatment option in hormone receptor-positive/HER2-negative metastatic breast cancer. CDK4/6 inhibitors are generally safe and manageable drugs, with a low rate of severe complications that could be overcame by dose control [291, 292]. Thus, CDK4/6 inhibitors offer an effective and tolerable treatment that can be combined with other therapies and thus harbours therapeutic potential for multiple cancers [233, 279, 280].

Understanding the clinical and molecular determinants of drug efficacy is paramount to improve the efficacy of anticancer and antiviral treatments. Based on our findings, the development of robust SAMHD1 inhibitors and activators able to potentiate antimetabolite therapeutic regimens against cancer, viral infections or other diseases should become a priority. In summary, this thesis highlights the importance of understanding intrinsic mechanisms of immune regulation to identify key proteins such as CD32 and SAMHD1, that are fundamental in the outcome of viral infections and the response to chemotherapy and antiviral agents. Indeed, a better understanding of the crossroad between HIV-1 latency, nucleotide metabolism and antimetabolite therapy could be crucial for the progression of human diseases and for the development of novel combinatory therapeutic strategies against HIV infection and cancer.

CONCLUSIONS

1. CD32 expression is upregulated upon T cell activation or *in vitro* infection with HIV-1 virus and correlates with cell proliferation marker Ki67 and T cell activation markers HLA-DR and CD69, indicating that CD32 expression is indicative of T cell activation.

2. CD32 is not a key marker of HIV latency neither in *in vitro* models of latent infection, nor in HIV-1+ patients. Presence of latent HIV provirus was not enriched in CD32 expressing lymphocytes and HIV-1 virions recovered after *ex vivo* stimulation of patient lymphocytes were similarly infectious in CD32+ and CD32- cell subsets.

3. Measurement of the anti-HIV-1 activity of anticancer drugs in primary macrophages is a reliable and highly sensitive approach to evaluate efficacy of drugs modulated by SAMHD1 function.

4. SAMHD1 function is able to either enhance or reduce the efficacy of anticancer drugs affecting nucleotide metabolism, including nucleotide analogues and anti-folate drugs. SAMHD1 substrates showed enhanced activity upon SAMHD1 degradation, whereas non-substrates presented limited efficacy in the absence of SAMHD1, as a result of increased intracellular dNTP pool.

5. Pharmacological activation of SAMHD1 function by CDK4/6 inhibitors is able to enhance the antiviral and cytotoxic efficacy of SAMHD1 non-substrates in primary macrophages and in cancer cell lines, representing a novel approach for the development of novel combinatorial anticancer therapeutic strategies.

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LIST OF PUBLICATIONS

1. **Castellví M**, Felip E, Ezeonwumelu IJ, Badia R, Garcia-Vidal E, Pujantell M, Gutiérrez-Chamorro L, Teruel I, Martínez-Cardús A, Clotet B, Riveira-Muñoz E, Margelí M, Ballana E. Pharmacological Modulation of SAMHD1 Activity by CDK4/6 Inhibitors Improves Anticancer Therapy. Cancers (Basel). 2020 Mar 18;12(3):713. doi: 10.3390/cancers12030713. Erratum in: Cancers (Basel). 2020 Jun 29;12(7): PMID: 32197329; PMCID: PMC7140116.

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