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"Play it again, SAMHD1": the hard life of an antiviral restriction factor during the intrinsic immune response against human cytomegalovirus infection.

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Summary

Sterile α-motif and histidine-aspartate domain-containing protein 1 (SAMHD1) is a cellular deoxynucleotide triphosphates (dNTPs) triphosphohydrolase that hydrolyses dNTPs into deoxynucleosides and inorganic triphosphates. This activity is associated with its antiviral restriction function and it inhibits replication of many RNA and DNA viruses. The antiviral activity is negatively regulated by Thr-592 phosphorylation, that destabilizes the active tetrameric form of the protein. Human cytomegalovirus (HCMV) is an opportunistic pathogen in immunocompromised hosts, while it is asymptomatic in the general population. Infection is life-long, and latency is a pivotal feature of the virus, which interferes with host anti-viral responses, including intracellular restriction factors. During my PhD project, we investigated the role of SAMHD1 in HCMV replication and potential viral evasion mechanisms, which could contribute to the unsuccessful clearance of the virus. After infection of different cell types with different HCMV strains, we observed an increase in SAMHD1 mRNA and protein levels. This was associated with the induction of phosphorylation at the regulatory residue Thr-592, likely involving the cellular kinase Cdk1, but not the viral kinase pUL97. Both SAMHD1 knock-down and, on the other hand, overexpression of wild-type and Thr-592 mutants showed negligible influence on HCMV viral production, suggesting that SAMHD1 activity could be overcome by HCMV during lytic infection, independently from its phosphorylation status. We also observed, by various experimental approaches, phospho-SAMHD1 localization in the cytoplasm of infected fibroblasts and its association with viral particles, suggesting the idea of a mechanism of de-localization and evasion from its nuclear-associated antiviral activity. Despite recent observations of SAMHD1 restriction during early steps of HCMV replication, our work suggests that SAMHD1 is unable to limit viral lytic infection, probably due to re-localization in the cytoplasm, setting a cellular environment permissive for a productive HCMV replication.

1. Introduction

1. HCMV

a. General characteristics

Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus that latently infects most of the world's population (Mocarski et al., 2013). It is a double strand DNA (dsDNA) virus, belonging to the β-herpesvirinae subfamily and it has the largest genome of all known human viruses, with a length of 235 kbp. *In vitro* and *in vivo* infections result in a characteristic cytopathology of enlarged (cytomegalic) cells, that gives to this virus its name (Britt, 2011). Like other human herpesviruses, HCMV clearance is never complete and it remains latent during the entire life of the host. Primary infection does not cause severe illness in the general immunocompetent population, except for an occasional febrile illness as well as infectious mononucleosis, but it is an important opportunistic pathogen in immunocompromised hosts (i.e. AIDS patients or transplant recipients). Susceptibility to HCMV infection is particularly related with defects in $CD4^+$ and $CD8^+$ T cell functions in immunocompromised or not fully competent hosts (Mocarski et al., 2013). For all these reasons, the virus remains clinically important despite antiviral therapies aimed at reducing disease burden. The clinical spectrum of HCMV infection of congenitally infected children, for example, involves numerous organs and tissues and comprises sensorineural hearing loss, microcephaly, splenomegaly, pneumonitis and even death (Britt, 2011). At cellular level, HCMV infects various cell types: monocytes, macrophages, neuronal, retinal, epithelial, endothelial and dendritic cells (DCs) (Revello and Gerna, 2010). Viral latency occurs in bone marrow-derived hematopoietic cells, where viral DNA is present at a very low copy number and it is associated with low viral transcription (Bego and St. Jeor, 2006).

As a result of long-standing infections, many genetically different strains of HCMV evolved and spread in the general population (Bradley et al., 2008), through contact with body secretions (e.g. urine, milk, saliva and uterine secretions). Breastfeeding is the most common way of mother-to-child transmission, but the transplacental one is the most

clinically important, and it occurs in women who are already infected before conception, as well as in women who experience primary infection during pregnancy (Britt, 2011).

b. Virion structure

The HCMV virion consists of three different regions: the envelope, the tegument and the capsid containing the viral genome (**Figure 1**). The envelope is composed of a variety of proteins that are largely still unknown in structure and function. Analysis of viral genome sequences indicated that almost 50 viral open reading frames (ORFs) can be predicted as membrane glycoproteins, and despite the exact number of envelope glycoproteins is still unknown, at least 12 different glycoproteins were identified (Britt and Boppana, 2004). Many of them (gB, gH, gL, gM and gN) have homolog function and structure in other herpesviruses, and they can exist as complexes hold together by disulfide bonds located in the portion of the protein inside the virion (gM/gN, gH/gL/gO and gH/gL/UL128/UL130/UL31A) (Britt and Boppana, 2004). gB is encoded by viral UL55 ORF and is expressed as a trimer, named glycoprotein complex I (gcI), and it mediates attachment to the plasma membrane, fusion and entry. The gcI trimer is important for virus spread and cell-to-cell membrane fusion, leading to formation of cellular syncytia (Mocarski et al., 2013). Several putative gB receptors were described: cell surface integrins (Feire et al., 2010), the epidermal growth factor receptor (EGFR) (Wang et al., 2003) and platelet-derived growth factor receptor alpha (PDGFRα) (Soroceanu et al., 2008). Nevertheless, the question of receptor requirement for virus entry is still under debate, as it has been reported that gB does not need cellular receptors during the membrane fusion (Wille et al., 2013). gM and gN glycoproteins are linked by disulfide bonds and form a heterodimeric complex named gcII. These proteins are encoded by UL100 and UL73 ORFs respectively, and they are the most abundant envelope glycoproteins (Nguyen and Kamil, 2018). The cytoplasmic tail of gM is essential during the stage of virion assembly (Krzyzaniak et al., 2007), while gN cytoplasmic tail is essential at the envelopment stage (Mach et al., 2007). Notably, the UL73 gene (gN) has one of the most variable sequences among different HCMV strains (Pignatelli et al., 2003). Moreover, gcII complex is a target of adaptive immune responses (Shimamura et al., 2006).

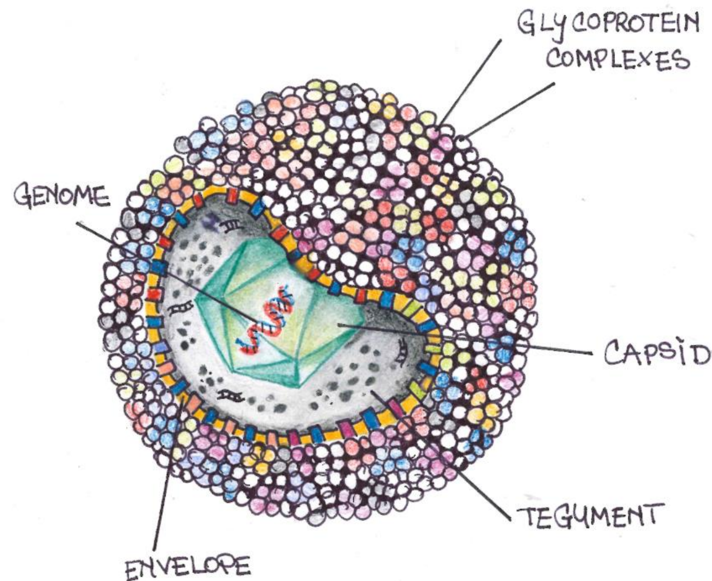

Figure 1: HCMV virion structure
HCMV structure consists of an outer bilayer envelope, composed of many glycoprotein complexes. Inside the envelope, there is the tegument, composed by proteinaceous matrix, which cover the icosahedral capsid containing the double stranded linear DNA genome.

gH, gL and gO form the heterotrimeric complex gcIII, and each protein is encoded by UL75, UL115 and UL74 ORFs, respectively (Nguyen and Kamil, 2018). PDGFRα acts as a receptor for the trimer and its role in viral entry is independent from its tyrosine kinase activity (Wu et al., 2018). The HCMV pentameric complex is composed by gH and gL heterodimer bound with *UL128*, *UL130* and *UL131* gene products (Ryckman et al., 2008). The pentamer has been described as necessary for infection of many cell types, including leukocytes, epithelial and endothelial cells (Adler, 2006). A putative cellular receptor binding the HCMV envelope pentamer is neuropilin-2, essential for attachment and entry in endothelial and epithelial cells (Martinez-Martin et al., 2018).
The tegument is the most complex and heterogeneous structure of HCMV virion. The tegument is composed by many viral proteins and RNAs and it is generally assumed as an amorphous layer between the envelope and the capsid (Chen et al., 1999). HCMV can include cellular proteins in its tegument and this strategy can be exploited by the virus to seize host anti-viral proteins, such as those mediating the intrinsic innate immune response (Dell’Oste et al., 2014). Tegument proteins are usually phosphorylated and many of them have a regulatory function in HCMV replication. In addition, they can regulate various

pathways in an infected cell: block of intrinsic cellular responses, stimulation of cell cycle progression, enhancement of immediate-early (IE) transcription and help in viral DNA replication (Baldick et al., 1997; Hayashi et al., 2000). One of the most abundant viral protein in the tegument is pp65, encoded by UL83 ORF, which is highly immunogenic: indeed, a percentage ranging from 2 to 5% of $CD8^+$ T lymphocytes in the peripheral blood of healthy seropositive individuals are specific for this protein (Khatamzas et al., 2002).
The capsid is the inner structure of the virion and consists of 162 capsomere subunits, divided in 150 hexons and 12 pentons built together in an icosahedral symmetry (Chen et al., 1999). The capsid is partially assembled in the cytoplasm, guided by viral UL80a ORF, that acts as a scaffold for the generation of individual capsomeres (Gibson, 2008). Capsids containing viral dsDNA exit the nucleus and are enveloped in the cytoplasm.

c. Genome structure and variability

The HCMV genome is one of the largest of all human viruses and is composed of two main coding domains, the unique long (UL) and unique short (US) domain. They are flanked in the outer and inner side of the genome by the long and short terminal repeated (TRL, TRS) and internal repeated (IRL, IRS) sequences (Bankier et al., 1991) (**Figure 2**).

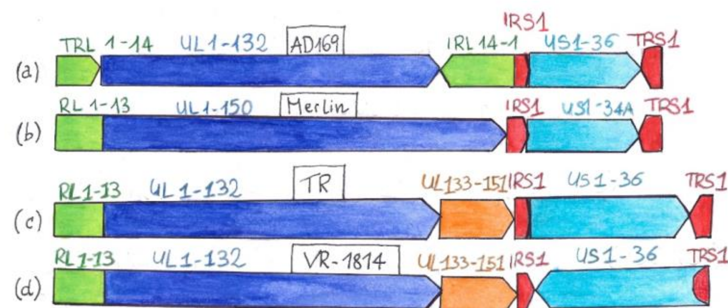

Figure 2: HCMV laboratory and clinical strain genomes

Organization of AD169 (a), Merlin (b), TR (c) and VR-1814 (d) genome. (a) AD169 genome comprise two unique blocks of ORFs (UL and US), each one flanked by repeated blocks of ORFs (TRL, IRL; IRS, TRS) (b) In Merlin genome, TRL and IRL blocks of ORFs are missing, the UL segment is larger and contain RL1-14 and UL1-151. (b)(c) TR and VR-1814 clinical isolates, after the unique domain RL1-13 and UL1-150, contain a block of genes (UL133–UL151) separated from the UL segment, and the US1-36 region is inverted in VR-1814 genome (adapted from Murphy et al., 2003)

The genome is tightly packaged inside the nucleocapsid and it circularizes during the replication phase of the virus, in a mechanism called "rolling circle amplification", that can generate multiple copies of the genome, linked in tandem (Gibson, 2008).

An extensive analysis of protein encoded by the well-known AD169 laboratory strain genome was conducted for the first time in 1990. The authors reported that AD169 HCMV laboratory strain contains 208 encoding ORFs and 14 of them are duplicated in TRL and IRL regions (Chee et al., 1990). Because of AD169 adaptation to *in vitro* cell culture conditions, AD169 completely misses genes from UL133 to UL151, encoding immune evasion proteins (Prichard et al., 2001).

Merlin strain has been extensively studied and its genome has been used as a reference wild-type genome (Martí-Carreras and Maes, 2019). Merlin has been also designated from the World Health Organization (WHO) as the international standard model for HCMV (Wilkinson et al., 2015). In 2004, Merlin strain was predicted to code for 165 protein (Dolan et al., 2004), but later research identified additional transcripts and more coding region could thus exist (Gatherer et al., 2011). In the same study, AD169 has been used as a model of high-passaged laboratory strain, and VR-1814 and TR strains were used as low-passaged clinical strain models. Compared to AD169, VR-1814 and TR are characterized by less DNA mutations and by the presence of the UL133-UL151 region (Murphy et al., 2003). At the present time, more than 300 full-length complete viral low and high passaged strain genomes have been published and it is estimated that HCMV contains ~ 751 translated ORFs (Martí-Carreras and Maes, 2019; Stern-Ginossar et al., 2012).

The severity of clinical outcomes caused by HCMV could be related to viral genetic variability, that has been shown to be remarkably high inside and between hosts (intra- and interhosts variability) (Renzette et al., 2015). As a consequence of HCMV ability to infect many different cell types and organs, viral populations sequenced from different tissue samples in the same individual can be as different as viral populations sequenced from distinct individuals (Renzette et al., 2013). It has been proposed that immune responses can trigger a selective pressure on HCMV during infection, favouring the selection of viral populations containing mutations advantageous for the successful spreading in the hosts (Renzette et al., 2011). Regarding interhost variability, it has been recently reported that viral strains freshly isolated from congenitally infected children are genetically and

phenotypically different, and characterized by different aggressiveness in models of *in vitro* infection (Galitska et al., 2018).

d. Virus entry, replication and latency

HCMV entry is very promiscuous and it can bind, penetrate and start replication in almost all cell types. This characteristic was originally explained with the hypothesis that the receptor for HCMV is distributed on the surface of a great variety of cells (Nowlin et al., 1991). In 1993, Compton and colleagues reported that virus entry requires the presence of the polysaccharide heparan sulfate on the cell surface, which is expressed in almost all cells (Compton et al., 1993). However, from a biochemical point of view, Boyle and Compton reported that HCMV can engage other molecules than heparan sulfate (Boyle and Compton, 1998). One of them was identified in 2003 to be the epidermal growth factor receptor (EGFR) (Wang et al., 2003). In their paper, the authors reported the co-immunoprecipitation of gB and EGFR after chemical crosslinking, but they did not resolve the exact mechanism of interaction between these two proteins. After this important finding, in the following years other HCMV receptors were reported: αvβ3 integrin (Wang et al., 2005), the platelet-derived growth factor receptor (PDGFR) (Soroceanu et al., 2008), and CD147 for the entry into epithelial and endothelial cells (Vanarsdall et al., 2018). OR14I1 and neuropilin-2 have been identified as receptors for the viral pentameric complex and responsible for the epithelial/endothelial tropism (Martinez-Martin et al., 2018; Xiaofei et al., 2019). After plasma membrane binding, regardless the receptor, the virus can entry the cell in a pH-independent manner through macropinocytosis, like in fibroblasts (Li et al., 2015a), or through endocytosis in a low pH-dependent manner, like in epithelial and endothelial cells (Ryckman et al., 2006). Then, the envelope/cell membrane fusion results in activation of several cellular signaling pathways and start of the viral lytic cycle (**Figure 3**).

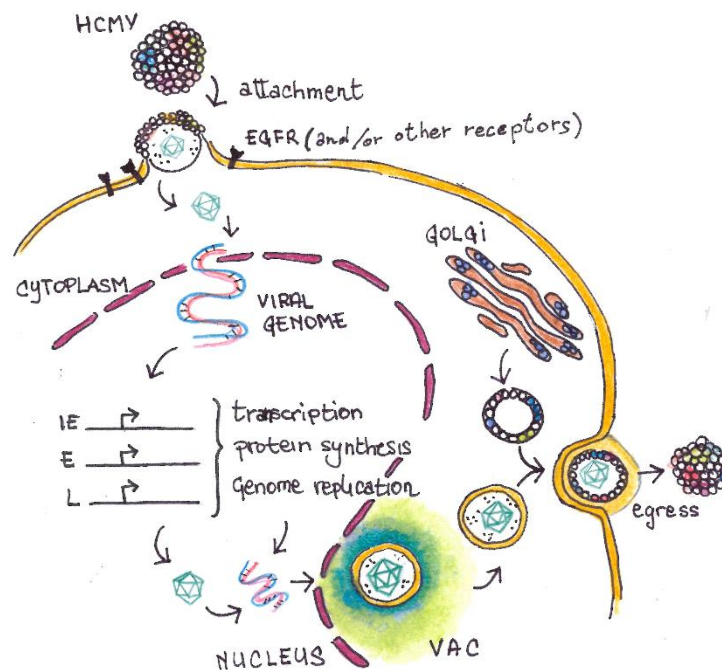

Figure 3: Overview of the HCMV lytic cycle
HCMV enters the cell through interaction with EGFR and other cellular receptors. Capsid is delivered into the cytoplasm and travels to the nucleus, where the genome is transcribed and replicated. The expression of viral genes follows three temporal moments: expression of immediate early (IE), early (E) and late genes (L). In the nucleus, late genes expression set up capsid assembly. Capsid translocates to the cytoplasm, associates with tegument components and is trafficked to the viral assembly complex (VAC). Subsequently, the capsids acquire viral envelope by budding into intracellular vesicles. After envelopment, viral infectious progeny is then released outside the cell.

HCMV lytic cycle can be divided in three distinct phases, characterized by the expression of different viral proteins, named immediate early (IE), early (E) and late (L) proteins, in relation to different stages of expression. After entry, the capsid is transported into the nucleus and the *IE1* and *IE2* genes are expressed without any active viral protein synthesis. Some viral proteins could be already present inside the virion, and host transcription factors are probably involved in the early induction of viral protein synthesis (Britt and Boppana, 2004). *IE1* and *IE2* genes result from transcription of the same viral genome region, the major immediate-early (MIE) gene and the resulting proteins derive from alternative splicing of the primary polyadenylated transcript. IE1 is a 72 kDa phosphoprotein detectable at very early times post-infection, while IE2 is an 86 kDa trans-activating factor of viral early and late genes. Together, IE1 and IE2 autoregulate their

expression, activate cellular genes, establish nuclear sites of lytic viral DNA synthesis and contribute to the latency/reactivation switching (Mocarski et al., 2013).

The MIE gene is located inside the so-called major immediate-early promoter (MIEP), in turn controlled by MIEP enhancer, that requires, for its regulation, the activity of tegument proteins pp71 and ppUL69. After MIE protein expression, the virus expresses early genes (E), that are important for alteration of cell cycle progression and apoptosis, for viral DNA transcription and production of structural virion proteins. Early genes comprise almost 65 proteins and a set of miRNAs that accumulate and act through 18 to 24 hours post-infection (h.p.i), at the time of viral DNA synthesis. During this time, the virus expresses late genes (L), that encode virion structural proteins and are required for the assembly of infectious particles (Mocarski et al., 2013).

DNA synthesis occurs inside the nucleus of infected cells starting at 14 h.p.i and reaching more than 10,000 copies from 24 to 48 h.p.i. The mechanism of synthesis starts from the oriLyt site and viral DNA replication occurs in a rolling circle dynamic of replication, leading to the formation of a long linear concatemer of multiple copies of HCMV genome that are packaged into pre-formed capsids inside the nucleus (McVoy and Adler, 1994). The replication start is dependent on viral replisome formation, composed by the viral UL54 DNA polymerase core subunit, UL44 DNA polymerase processivity subunit, UL57 DNA single strand-binding protein and the viral helicase-primase heterotrimeric complex (Mocarski et al., 2013). DNA polymerase UL54 core subunit is the target of the antiviral drugs ganciclovir and foscarnet, and mutations in *UL54* gene sequence have been described to contribute to antiviral drug resistance (Chou et al., 2003). Capsid localization, packaging and cleavage of viral DNA are positively regulated by phosphorylation from pUL97 kinase and cellular cyclin-dependent kinases (Cdks). The exit of the capsid from the nucleus is mediated by a herpesvirus conserved nuclear egression complex (NEC) localized in the nuclear inner membrane. NEC acts as a mechanism of packaging quality control of DNA-containing capsids. During this phase, the nucleocapsid temporary envelopes from the nuclear inner membrane that is subsequently lost in the cytoplasm (Britt, 2011). After this stage, the nucleocapsid reaches a perinuclear area known as virus assembly complex (VAC), where the virus acquires the tegument and it is enveloped from endosomal/exosomal membranes before the final egress from the cell (Mocarski et al., 2013) (**Figure 3**).

Latency is a characteristic of all herpesviruses, and HCMV is not an exception. Latent infection has been shown in cells of the myeloid lineage, such as $CD34^+$ progenitors, macrophages and $CD14^+$ monocytes (Hahn et al., 1998). HCMV can reactivate upon differentiation of these cell types, and mature circulating DCs and macrophages were reported to be sites of reactivation (Poole et al., 2015; Reeves and Sinclair, 2013). Despite the mechanisms that favor the establishment of latency are still largely unknown, a hallmark of this process is the suppression of MIEP activity and, subsequently, one of the first events of reactivation is the production of IE proteins. Given that, MIEP activity can be regulated by transcription factors and epigenetic/chromatin structure modifications (Sinclair, 2009; Stinski and Isomura, 2008). In particular, the latent genome has a MIEP chromatin structure associated with transcriptional repression, including trimethylation of Lysine-27 and Lysine-9 sites inside H3 histone. In addition, the activity of histone deacetylase (HDAC) maintains viral chromatin in a repressed conformation (Elder and Sinclair, 2019). Indeed, treatment of latently infected cells with HDAC inhibitors is associated with reactivation of IE gene transcription (Krishna et al., 2018).

e. HCMV and cell cycle regulation

HCMV can subvert and modify host cell cycle to favor its own replication and the establishment of a lytic or latent infection. The cell cycle is tightly regulated by the activity of cyclins and cyclin-dependent kinases (Cdks), that form active heterodimers and phosphorylate serine and threonine residues of target proteins, to obtain their activation or inactivation. Human cells express about 20 Cdks and 13 cyclins to regulate transition through the cell cycle, but also to control genetic transcription, epigenetic modifications, metabolic activity and DNA repair. Moreover, the activity of Cdks and cyclins are intertwined with cell cycle checkpoints, that are essential to ensure a correct progression during the multiple phases of the cycle (Roskoski, 2019).

One of the main characteristics of HCMV-infected cells is the arrest of cell cycle before mitosis, and it is particularly interesting the fact that they express G_1, S and M-phase-associated proteins but are blocked in DNA synthesis (Spector, 2015). Indeed, HCMV influence on cell cycle mechanisms is very deep: it influences expression, translation, post-translation modifications, stability and localization of cellular proteins. For example, the

viral product pUL97, a serine/threonine kinase, can hyper-phosphorylate Rb to release E2F and allow active gene transcription (Jault et al., 1995). IE1 and IE2 proteins are able to modify cell cycle progression in different ways: IE1 can stimulate entry into the S phase removing the p107 repression of E2F promoters, and IE2 can support p53 expression and accumulation in the nucleus and entry into S-phase (Castillo et al., 2000). Moreover, the virus targets the anaphase promoting complex (APC), to modulate its activity and avoid degradation of thymidine kinase (TK) and ribonucleotide reductase (RNR), to ensure the production of enough nucleotide triphosphates for viral genome replication (Fehr and Yu, 2013).

Thus, cell cycle manipulation is an important strategy carried out by the virus to stimulate its own DNA replication and disadvantage synthesis of host DNA, starting already at early times post-infection.

f. Immune responses

In hosts with an intact and fully competent immune system, innate and adaptive immunity can limit HCMV spread in the organism and prevent the arise of serious illness. Studies in experimental murine models helped to delineate the role of these two arms of the immune system in controlling virus replication, and the key importance of DCs, natural killer (NK) cells, $CD4^+$ T helper cells and $CD8^+$ cytotoxic T lymphocytes (CTL), interferon responses and virus-specific antibodies (**Figure 4**). In immunocompromised hosts, defects of innate and adaptive immune responses predispose to HCMV infection, resulting in an increasing probability of morbidity and mortality (Britt, 2011).

The main characteristic of the immune responses in immunocompetent individuals is a robust CTL and neutralizing antibody response to the infection. Despite that, the control of the virus is not complete, and clearance is impossible. This is largely due to the expression of viral gene products that modulate and disrupt host innate and adaptive immune responses, establishing a life-long balance between immune-evasion mechanisms of the virus and the immune system that tries to eradicate the pathogen.

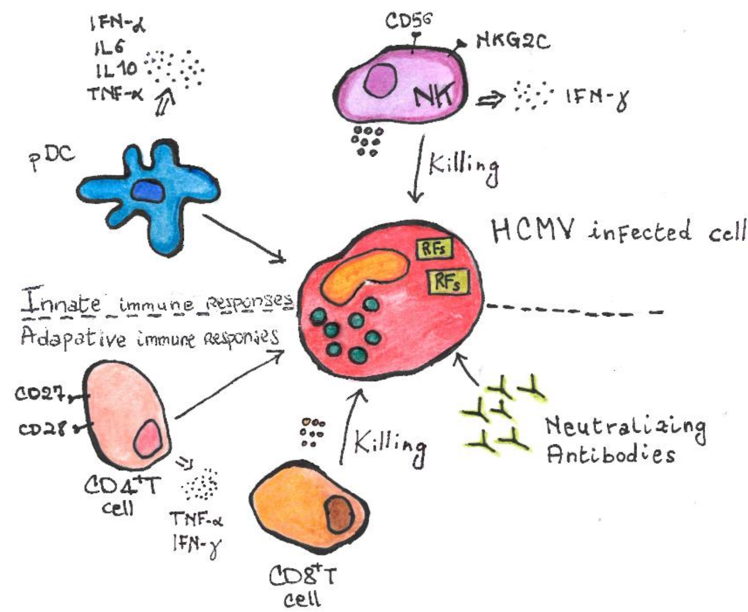

Figure 4: HCMV immune responses

Schematic representation of immune cells and their effector mechanisms against HCMV-infected cells. Intracellular restriction factors (RFs) interfere with many steps of HCMV replication. pDCs release IFN-α, IL-6, IL-10 and TNF-α to mediate humoral and cell-mediated immune responses. NK cells limit HCMV producing IFN-γ and killing infected cells by secretion of cytotoxic granules. Neutralizing antibodies target viral epitopes preventing cell attachment, penetration and HCMV dissemination. $CD8^+$ T cells release cytotoxic granules to kill HCMV-infected cells. CD27 and CD28 double positive $CD4^+$ T cells produce IFN-γ and TNF-α, support $CD8^+$ T cell persistence and maintain viral-specific antibody production.

The very first line of defence against viral pathogens is grouped in the innate arm of the immune system, known as “intrinsic immunity”, that involves various proteins that act as defence mechanisms at the cellular level. The most characterized and studied intrinsic immune responses are accomplished by cellular “restriction factors” (RFs), constitutively expressed intracellular proteins able to interfere with many steps of viral replication (Bieniasz, 2004). Because RF response to HCMV infection is the focus of this thesis, it will be discussed in a separate paragraph later in the text (see page 20).

NK cells are key components of innate immune responses. Individuals with NK cell defects are susceptible to herpesvirus infections, including HCMV (Biron et al., 1989; Gazit et al., 2004). In general, one of the main features of NK cell responses to infected cells is the release of proteins with cytotoxic activity, leading to a rapid killing of infected cells (Santoli et al., 1978). NK cells can also produce cytokines and chemokines, to modulate inflammatory responses and to recruit various effector cells to the site of infection (Fehniger et al., 1999; Loza et al., 2002). NK cell activation, proliferation and

effector functions depend on a balance between positive signals provided by many activating receptors (i.e. NKG2D, NKG2C, NKp46) and negative signals promoted by inhibitory receptors (i.e. KIRs, NKG2A) interacting with major histocompatibility complex (MHC) class I molecules (Lanier, 2005). Moreover, HCMV infection triggers an enrichment of a particular NK cell subset: adult hematopoietic stem cell recipients experimenting HCMV reactivation from latency, as well as infected children, show an expansion of a mature $CD56^{dim}$ $CD16^{-}$ NK cell subset expressing NKG2C, probably involved in the control of HCMV reactivation (Goodier et al., 2018; Gumá et al., 2004). Furthermore, NK cells can limit HCMV transmission in epithelial, endothelial cells and fibroblasts, directly producing IFN-γ and inducing IFN-β production in these infected cells (Iversen et al., 2005; Wu et al., 2015).

At the same time, DCs are also important players in the innate immune responses against pathogens. They are crucial for the efficacy of antiviral responses, by stimulating chemokine and cytokine production to limit viral dissemination, and by triggering the activation of adaptive immune responses. During HCMV infection, plasmacytoid DCs (pDCs) are particularly important, because they are non-permissive to viral HCMV replication, but remain functional to facilitate the mounting of an effective immune response (Varani et al., 2007). For example, experiments conducted on pDC isolated from human peripheral blood mononuclear cells, showed that HCMV infection triggers IFN-α, IL-6, IL-10 and TNF-α production by pDCs, critical for humoral and cell-mediated immune responses (Varani et al., 2007).

Immunocompetent individuals infected with HCMV, show a high proportion of $CD4^{+}$ T cells that recognize pp65 and IE-derived immunodominant epitopes (Jackson et al., 2011). During primary infection of kidney transplant recipient, $CD4^{+}$ T cells specific for HCMV epitopes appear approximately 7 days after detection of viral DNA in peripheral blood (Rentenaar et al., 2000). Moreover, during the acute phase of infection, these cells are CD27 and CD28 double positive and produce IFN-γ and TNF-α cytokines (Rentenaar et al., 2000), while in the latent phase, these cells are prevalently CD27 and CD28 double negative and acquire a cytolytic function, characterized by the expression of perforin and granzyme B (van Leeuwen et al., 2006).

Together with $CD4^{+}$ T cell responses, CTL responses emerge and persist also during the latent phase of infection, and are crucial for HCMV control of reactivation (Kuijpers et al.,

2003). In bone marrow transplantation recipients, regain of CTL populations protects from HCMV-associated disease (Avetisyan et al., 2007). During viral persistence, impairment of $CD8^+$ T cell maturation and effector functions contributes to the failure of the immune system in controlling viral replication and disease (Gamadia et al., 2003). Over the entire life of infected hosts, a high number of memory $CD8^+$ T cells specific for HCMV expand with aging and persist in the circulation. This peculiarity is also named *memory inflation*, characterized by memory $CD8^+$ T cells expressing IL-7 receptor and thus responding to IL-7 mediated homeostatic proliferation (Waller et al., 2008). Moreover, during *memory inflation*, $CD4^+$ T cells contribute to a lesser extent in controlling HCMV reactivation, compared to $CD8^+$ T cells, sharing the characteristic of IL-7 receptor-dependent expansion and persistence at aging of infected individuals (Gamadia et al., 2004; Libri et al., 2011).
During HCMV infection, many specific neutralizing antibodies are produced, preventing cell attachment, entry and dissemination in the organism. Confirming the importance of HCMV envelope glycoprotein complexes for viral entry in host cells, gB and gH (components of the gcI, gcIII and pentameric complexes) are two of the main immunogenic proteins encoded by HCMV targeted by neutralizing antibodies (Mocarski et al., 2013), together with gM/gN, components of gcII complex (Shimamura et al., 2006). Moreover, specific antibodies can also target the tegumental protein pp150 as well as the DNA polymerase processivity factor subunit pp52 (encoded by UL44 gene) (Beqaj et al., 2008; Greijer et al., 1999). The role of anti-HCMV antibodies can be deducted from studies in B-cell deficient mouse models and from correlation of these studies in humans, recapitulating the importance of antibodies in limiting the severity of HCMV-related disease but not to prevent infection and viral dissemination. However, to date, a clear evidence demonstrating the effectiveness of anti-HCMV neutralizing antibodies in protecting humans from viral reactivation and spread still lacks (Krmpotić et al., 2019).

g. HCMV and immune evasion

HCMV can efficiently establish a latent infection in the host and this reflects its ability to evade immune responses. In fact, the genome encodes various immune evasion proteins that can target different molecular pathways and cells of the immune system (**Table 1**).

Among innate responses, HCMV can influence NK cell recognition of target cells in different ways. The UL18 viral glycoprotein, an HLA-I homolog, can bind to the inhibitory receptor leukocyte immunoglobulin-like receptor-1 (LIR-1) (Cosman et al., 1997) and suppress LIR-1^{+} NK cell activation (Prod'homme et al., 2007). In a similar way, a peptide encoded by the viral UL40 protein can upregulate HLA-E expression on target cells, which is recognized by the inhibitory complex CD94/NKG2A on NK cells and thus inhibit NK cell-mediated lysis (Cerboni et al., 2001; Ulbrecht et al., 2000). Other HCMV-encoded NK cell modulators comprise RL11, UL119-118, UL148, all implicated in the reduction of NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC), and the NK cell activating ligand modulators UL16, UL148A, US9, US18, US20, UL141 and UL142, that downregulate the ligands of the activating receptors NKG2D and DNAM-1 (Patel et al., 2018).

HCMV can also compromise DC function. Studies of HCMV interaction with DCs focused mainly on monocyte-derived dendritic cells (MDDCs), useful for their characteristic to be easily stimulated *in vitro*. HCMV can productively infect MDDCs, by the binding of its envelope protein gB to the membrane protein DC-SIGN (Halary et al., 2002). Infected MDDCs were reported to have an impaired production of IL-12 and TNF-α in response to LPS stimulation (Moutaftsi et al., 2002), and they can suppress T lymphocyte priming and proliferation and induce their CD95L- and TRAIL-mediated apoptosis (Haspot et al., 2012; Raftery et al., 2001). HCMV can also impair MDDC chemotaxis to lymph nodes (Gredmark-Russ and Söderberg-Nauclér, 2012). The impaired migratory ability has been associated with a reduction of the C-C chemokine receptors 1 and 5 (CCR1 and CCR5) expression by MDDCs (Varani et al., 2005).

The US2, US3, US6, US10 and US11 viral products can modulate and interfere with surface expression of cellular MHC class I and II and antigen presentation in different ways, resulting in evasion from CD8^{+} and CD4^{+} T cell recognition and activation, respectively, and causing a severe impairment of adaptive immune responses (Jackson et al., 2011; Noriega et al., 2012). In particular, US3 acts during the immediate early phase of infection, retaining MHC class I molecules in the endoplasmic reticulum (ER) (Jones et al., 1996). During the early phase, US2 and US11 can induce ubiquitination and degradation of MHC class I or retain the heavy chains in the ER, blocking MHC-peptide complex formation and expression on cellular surface (Furman et al., 2002; Shamu et al., 2001;

Tomazin et al., 1999). During late phases, US6 can inhibit TAP activity and translocation of processed peptides to MHC class I complexes in the ER (Ahn et al., 1997). Beside the influence on MHC class I expression, US2 and US3 have been reported to downregulate the expression of MHC class II molecules as well (Tomazin et al., 1999). In addition, the truncated UL111A transcript, expressed during latency, can downregulate MHC class II molecules on the surface of infected myeloid progenitors, reducing their ability to present antigens to $CD4^+$ T lymphocytes (Cheung et al., 2009). At the same time, HCMV can interfere with the IFN-γ-mediated upregulation of MHC class II molecules through disruption of the Jak/STAT pathway and subsequent antigen presentation to $CD4^+$ T cells, limiting the activation and function of these cells (Miller et al., 1998).

HCMV is also able to counteract the specific antibodies produced against its immunogenic epitopes. To the best of our knowledge, the virus encodes four distinct glycoproteins which are homologues of the receptors for the Fc fragment of IgG (vFcγR): gp68, encoded by the *UL119-UL118* gene, gp34, encoded by the *TRL11/IRL11* gene (Atalay et al., 2002; Lilley et al., 2001), gpRL13 and gpRL12, encoded by RL11 gene family members (Cortese et al., 2012). The precise biological meaning of this strategy of immune evasion is unclear, but it has been proposed that these four glycoproteins, expressed on the surface of HCMV-infected cells, can bind and mask the Fc domain of host IgG antibodies, blocking complement fixation and activation, and preventing ADCC (Atalay et al., 2002).

Beside viral proteins, HCMV also encodes microRNAs (miRNAs) to prevent infected-cell recognition and clearing by the immune system (Ng et al., 2015). For example, viral miR-UL112 was the first to be bioinformatically predicted and then experimentally validated to target the NK cell-activating ligand MICB mRNA and to reduce its translation (Stern-Ginossar et al., 2007). Interestingly, miR-US25-2-3p targets tissue inhibitor of metalloprotease 3 (TIMP3), leading to activation of extracellular metalloproteases. Subsequently, active metalloproteases increase MICA and MICB shedding, reducing NK cell-mediated recognition and killing (Esteso et al., 2014). Viral miRNAs can also influence CTL responses, transcribing miR-US4-1 and miR-UL112-5p, that affect ER aminopeptidase 1 (ERAP1)-mediated antigen processing (Kim et al., 2011; Romania et al., 2017); miR-UL112-1, that modulates IL-32-mediated inflammatory responses (Huang et al., 2013) and miR-UL112-3p, that influences TLR-2 activity (Landais et al., 2015).

Table 1: HCMV immunomodulatory molecules (adapted from Patro, 2019)

HCMV products	Mechanism of evasion and effect on host immune system	Reference
UL18	HLA-I homolog; inhibition of NK cells	Cosman et al., 1997; Prod'homme et al., 2007; Cerboni et al., 2006
UL40	Up-regulation of HLA-E; inhibition of NK cells	Ulbrecht et al., 2000; Cerboni et al., 2001
RL11, UL119-118, UL40	Reduction of NK cell-mediated ADCC	Reviewed in Patel et al., 2018
UL16, UL148A, US9, US18, US20, UL141, UL142	Downregulation of ligands of the NK cell-activating receptors NKG2D and DNAM-1	Reviewed in Patel et al., 2018
US3, US2, US11, US6, UL111A	Downregulation of HLA-I and HLA-II expression; inhibition of $CD8^+$ and $CD4^+$ T cell recognition	Reviewed in Jackson et al., 2011
gp68, gp34, gpRL13-gpRL12	Viral FcR homologs	Lilley et al., 2001; Atalay et al., 2002; Cortese et al., 2002
miR-UL112	Reduction of MICB translation	Stern-Ginossar et al., 2007
miR-US25-2-3p	Reduction of TIMP3 expression; increase of MICA and MICB shedding	Esteso et al., 2014
miR-US4-1, miR-UL112-5p	Reduction of ERAP1 expression; negative regulation of antigen processing	Kim et al., 2011; Romania et al., 2017
miR-UL112-1	Negative regulation of IL-32 mediated inflammatory responses	Huang et al., 2013
miR-UL112-3p	Negative regulation of TLR-2 activity	Landais et al., 2015

HCMV can also influence the mechanisms of intrinsic immunity, comprising cellular RFs. Despite the many RFs known to inhibit HCMV replication, the virus can effectively replicate in host cells, suggesting an evolution of evasion strategy also from restriction activity. RF responses to HCMV infection are the focus of this thesis work and will be extensively described in the next chapter.

2. Restriction factors

Antiviral immune responses comprise also those ones mediated by the intrinsic innate immune system. This is characterized by restriction factors (RFs), host proteins that limit viral infection and replication at the cellular level and target different steps of viral life cycle, from entry to replication and spreading in the organism. The cellular activities grouped as intrinsic immunity were first reviewed in 2004 by Paul Bieniasz and at that time, they comprised Friend virus susceptibility protein-1 (Fv1), Fv-like proteins (e.g. the TRIM family) and apolipoprotein B editing catalytic subunit-like (APOBEC) gene families (Bieniasz, 2004). Historically, research on intrinsic immunity pathways started in 1960s, focused on cellular responses to retroviral infections, and only later this field comprised a broader set of viral pathogens, including HCMV. From the early 1970s, when the cellular Fv1 protein was described to protect mice from lethal doses of murine leukaemia virus (MLV), many research works aimed to describe the role of RFs during viral infections and, at the present time, many of them have been described. RFs are very different proteins that share some common characteristics: most are encoded from interferon stimulated genes (ISGs), are germline encoded and can be found in almost every cell type (Kluge et al., 2015). Notably, many RFs are constitutively expressed and ready to act during very early steps of infection (i.e. viral entry in the cell) and most interestingly, they can affect the virus specificity for target cells.

From a molecular and structural point of view, RFs are very different from each other. They contain diverse functional domains and they are active in various multimeric forms, attempting many different strategies (Chemudupati et al., 2019). For example, APOBEC3G was reported to restrict HIV-1 and HBV by deaminating cytidine on viral genome and inducing hypermutation, leading to prevention of viral reverse transcription (Bonvin et al., 2006; Sheehy et al., 2002). However, it was also reported to act in a deaminase-independent manner, binding the retroviral RNA template and sterically blocking reverse transcription (Iwatani et al., 2007). Or, as an example of non-enzymatic RF, IFN-induced transmembrane protein 3 (IFITM3) was reported to block virus entry and limit infection of influenza, West Nile, Dengue virus, and of HIV-1, probably limiting plasma membrane fluidity and preventing viral fusion (Brass et al., 2009; Li et al., 2013; Lu et al., 2011). In brief, frontline defences against viral pathogens can be obtained by

target cells with different means, and, more importantly, they target different steps of viral replication.

a. HCMV and restriction factors

Regarding HCMV infection, lysine-specific demethylases (KDMs), nuclear domain 10 (ND10) family proteins, viperin, absent in melanoma 2 (AIM2), IFN-γ inducible protein 16 (IFI16) and APOBEC3 proteins were reported to counteract and limit its replication, beside their activity against other viruses (Landolfo et al., 2016; Pautasso et al., 2018; Rossini et al., 2012) (**Table 2**). KDM activity limits HCMV latency establishment, by removing methyl groups on histone lysines associated with the MIEP, and subsequently stimulating the entry of the virus in the lytic phase of infection. In this context, the UL138 gene product can interfere with KDM activity in a yet undetermined mechanism, and can promote the establishment of latency (Lee et al., 2015). ND10 are dense bodies inside the nuclear matrix and are mainly composed by speckled 100 kDa (Sp100), human death domain-associated (hDaxx) and promyelocytic leukaemia (PML) proteins, that act in concert to regulate gene expression, DNA damage responses, oncogenesis and apoptosis (Rossini et al., 2012). HCMV IE1 protein can disrupt ND10 bodies formation and increase the efficiency of viral lytic replication (Korioth et al., 1996; Tavalai et al., 2006) but, at the same time, Sp100, hDaxx and PML proteins can restrict this mechanism of viral evasion, by silencing IE gene expression (Landolfo et al., 2016). Moreover, as a further countermeasure, the HCMV tegumental protein pp71 can interact with hDaxx, and target it for proteasomal degradation, thus relieving MIEP repression (Cantrell and Bresnahan, 2005).

Another RF known to affect HCMV replication is viperin, an IFN-inducible factor whose expression is induced by HCMV infection, among other viruses. It can disrupt plasma membrane lipid rafts, probably preventing the last phases of virion assembly and envelopment during HCMV lytic cycle (Chin and Cresswell, 2001). As viral countermeasure, HCMV relocates viperin from ER to mitochondria, where it negatively regulates ATP generation, disrupting cytoskeleton and sustaining viral infection (Seo et al., 2011).

Table 2: Restriction factors for HCMV (adapted from Landolfo et al., 2016)

Restriction factors	Effect on HCMV	HCMV countermeasure	Reference(s)
KDMs	Limit establishment of latency; stimulate lytic infection	UL138: prevents association of KDM with MIEP	Lee et al., 2015
ND10 (Sp100, hDaxx, PML)	Silence expression of IE genes	IE1: disrupts ND10 bodies formation pp71: targets hDaxx to proteasome	Korioth et al., 1996 Tavalai et al., 2006 Cantrell et al., 2015
Viperin	Prevents virion assembly and envelopment	vMIA: binds viperin and translocates it to mitochondria	Chin and Cresswell, 2001 Seo et al., 2011
IFI16	Senses viral DNA in the nucleus; inhibits viral UL54 promoter; binds and blocks Sp-1 transcription factor	pUL97: phosphorylates IFI16 and promotes its exit from the nucleus pp65: promotes IFI16 inclusion in the viral tegument	Gariano et al., 2012 Dell'Oste et al., 2014
APOBEC3 family	Deaminate cytidines and prevent transcription of viral genes	Under-representation of cytidines throughout the genome	Bonvin et al., 2006 Pautasso et al., 2018

Among other IFN-inducible factors, AIM2 and IFI16 are two proteins of the pyrin and HIN domain containing proteins (PYHIN) family, and they were initially described as pattern recognition receptors (PRRs) for intracellular viral DNA (Ansari et al., 2013; Man et al., 2016). IFI16 has been reported to block Sp1-like transcription factor binding to viral UL54 promoter (encoding the HCMV DNA polymerase), thus limiting viral DNA replication (Gariano et al., 2012). However, HCMV evolved a strategy of evasion from this important mediator of intrinsic immunity. In fact, pUL97 viral kinase phosphorylates IFI16, promoting its exit from the nucleus, the subsequent interaction with the viral pp65, resulting in the inclusion of this RF into the viral tegument (Dell'Oste et al., 2014).

As mentioned above, APOBEC3 family of proteins are cytidine deaminases that act on viral genomes and induce hypermutation, leading to prevention of viral genes transcription (Bonvin et al., 2006). APOBEC3A protein is upregulated during HCMV infection on maternal decidua and its overexpression has been reported to cause a delay of HCMV replication (Weisblum et al., 2017). In HCMV-infected fibroblasts, APOBEC3G and

APOBEC3F are upregulated after infection, in an IFN-β mediated manner. Interestingly, APOBEC3G overexpression or knock-down does not influence HCMV replication, suggesting a possible viral immune evasion mechanism. In fact, under the selective pressure of APOBEC proteins, HCMV evolved an under-representation of cytidines throughout its genome, to avoid the APOBEC-driven restriction (Pautasso et al., 2018).
As far as SAMHD1 is concerned, this is one of the most extensively studied RFs. It was firstly reported to limit HIV-1 infection, and then revealed to restrict also various RNA and DNA viruses. While this thesis was in preparation, in July 2019 “Cell Reports” published the first study of the role of SAMHD1 in HCMV infection, which appears to act in limiting the transcription of *IE* genes during the early phases of the infection (Kim et al., 2019). However, since the role of SAMHD1 during HCMV infection is the object of this thesis, SAMHD1 will be extensively described in the next chapter, and its implication in viral restriction will be discussed in detail.

3. SAMHD1

Sterile α motif (SAM) and histidine-aspartic domain (HD) containing protein 1 (SAMHD1) is a cellular deoxynucleotide triphosphates (dNTPs) triphosphohydrolase, that hydrolyses dNTPs into deoxynucleosides (dNs) and inorganic triphosphates (Goldstone et al., 2011). To date, it is not only one of the most known RFs against HIV-1, but it is the only cellular protein known to attend the triphosphohydrolase function and thus, the only one to negatively balance the activity of cellular ribonucleotide reductase (RNR), reducing the pool of available dNTPs for cellular and viral DNA synthesis.

a. Expression

SAMHD1 was discovered by Li and colleagues in 2000, as a human homologue of MG21, a mouse IFN-γ induced gene (Lafuse et al., 1995). In the article published by Li et al., SAMHD1 was firstly identified in an extensive screening of a cDNA library generated from human MDDC and was designated as dendritic cell-derived IFN-γ induced protein (DCIP) (Li et al., 2000).

Human *SAMHD1* gene locates on chromosome 20 and the protein is expressed by most cell types. Expression levels can vary among different tissues: for example, small intestine, spleen and ovary express SAMHD1 at high levels, but adipose tissue, liver and muscle express low or irrelevant amounts of the protein (Schmidt et al., 2015). Supporting its role as an antiviral RF of HIV-1, the expression profile in human cells shows an abundant and constitutive expression in vagina, foreskin and rectum tissue, sites of viral entry during sexual contact, as well as in leukocytes, resident and/or infiltrating these tissues. Moreover, cycling and non-cycling monocytes, macrophages, dendritic cells, $CD4^+$ and $CD8^+$ T cells, show high expression of SAMHD1 (Schmidt et al., 2015). Compared to proliferating primary human fibroblasts, the quiescent ones show an increased SAMHD1 expression, associated with a strong decrease of intracellular dNTPs (Franzolin et al., 2013).

Regarding SAMHD1 regulation of expression, $CD4^+$ T cell lines and lung adenocarcinoma samples were used to reveal mechanisms of epigenetic regulation of the promoter, that contains CpG islands prone to methylation and subsequent transcriptional repression (de Silva et al., 2013; Wang et al., 2014). SAMHD1 expression can be upregulated *in vitro* in primary macrophages by IL-12 and IL-18 treatment and in an IFN-γ-independent way, leading to restriction of HIV-1 (Pauls et al., 2013). In lung fibroblasts, SAMHD1 expression can be upregulated by TNF-α treatment and it is IRF-1 dependent (Liao et al., 2008). IFN-I and IFN-II stimulation of primary monocytes can induce SAMHD1 expression, downregulating miR-181a and miR-30a (negative regulators of SAMHD1 translation), with no changes in the promoter activity (Riess et al., 2017). On the other hand, $CD4^+$ T cells and DCs did not show an overexpression of SAMHD1 after IFN-I treatment, maybe due to SAMHD1 mRNA post-transcriptional regulation (St Gelais et al., 2012). SAMHD1 transcription can be induced also by phospho-IRF3, that directly binds SAMHD1 promoter and increases its expression after activation of intracellular PRRs (Yang et al., 2016).

b. Structure

SAMHD1 is a 72 kDa protein of 626 amino acids and it is characterized by the presence of two domains, the SAM and the HD domain, expressed in tandem (Liao et al., 2008) (**Figure 5**).

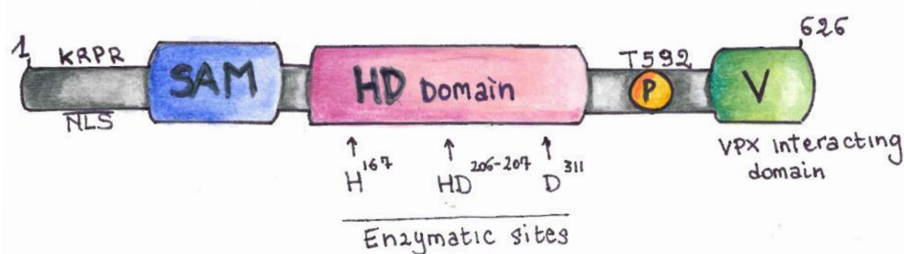

Figure 5: Schematic representation of SAMHD1 monomer

Starting from N-terminal portion (left), KRPR nuclear localization signal (NLS), the SAM and HD domain, T592 regulatory site and Vpx interacting domain are represented. The four enzymatic sites inside the HD domain are also shown (adapted from Sze et al., 2013).

The sterile α motif (SAM) domain is important for protein-protein interactions, it is dispensable for the triphosphohydrolase activity, but it is required for its maximal activity. SAM domain is so called because 4 out of 14 proteins identified by Ponting et al. that contain this domain are involved in yeast sexual differentiation, and it contains a secondary protein structure rich of α-helices (Ponting, 1995). In human cells, SAM domains are present in proteins with different biological functions: Tyr and Ser/Thr kinases, lipid kinases, scaffolding proteins, RNA binding proteins and transcription factors. In general, SAM domains are very much represented as protein-protein interaction domains (Qiao and Bowie, 2005).

The HD domain is responsible for the triphosphohydrolase activity (Beloglazova et al., 2013), exerted by three enzymatic sites, H^{167}, $HD^{206-207}$ and D^{311} (Sze et al., 2013). This domain is part of a superfamily of metal-ion-dependent phosphohydrolases rich of histidine and aspartic acid residues, that are evolutionary conserved in proteins involved in nucleic acid metabolism (Aravind and Koonin, 1998; Laguette and Benkirane, 2012). It extends from amino acid 162 to 335 and the crystal structure revealed the presence of both α-helices and β-barrel folds, that contain the key amino acids residues for its catalytic activity (Goldstone et al., 2011). The HD domain also provides the protein with the interface for enzyme oligomerization, binding dGTP and dNTPs to the allosteric sites, thus permitting the assembly of the tetrameric active complex (Powell et al., 2011) (see below and **Figure 7**). Notably, dNTPs, and particularly dGTP, are not only substrates of the enzyme but also allosteric activators, and this characteristic renders SAMHD1 both a regulator and a sensor of the cellular dNTP pool. Interestingly, this characteristic is shared

by the cellular enzyme RNR, responsible for dNTP production, thus acting on the other arm of the dNTP pool balance. Like SAMHD1, RNR is active in an oligomeric state, with allosteric sites that sense intracellular dNTP concentration and, in a similar manner, its activity is regulated by nucleotide binding (Ji et al., 2014).

SAMHD1 is generally described as a nuclear protein, and contains a nuclear localization signal (NLS) located on its N-terminal portion (Brandariz-Nuñez et al., 2012), although it was also reported a cytoplasmic localization in resting $CD4^+$ T cells (Baldauf et al., 2012). At the C-terminal portion, SAMHD1 contains a viral protein x (Vpx)-interacting domain, that is the site of interaction with HIV-2 and simian immunodeficiency virus (SIV)-encoded accessory protein Vpx (Laguette et al., 2012). Near the Vpx-interacting domain, there is the regulatory residue Threonine 592 (T592), an important site of phosphorylation and the most characterized mechanism of negative regulation of SAMHD1 activity and restriction (discussed later in the text, see page 29).

c. Metabolic activity and functions

As mentioned above, SAMHD1 is a regulator of dNTP pool and catalyzes the hydrolyzation of dNTPs into dN and triphosphates. This activity is tightly associated with three intertwined important functions of SAMHD1: cell cycle regulation, sensing of genome integrity and antiviral restriction (discussed later in the text, see page 33) (Mauney and Hollis, 2018). Substrate nucleotides allocate in the active sites and are stabilized by interactions with water molecules between them and the amino acids, leading to unspecific interactions that permit a “substrate promiscuity”. In the active sites of the HD domain, the core amino acids His167-His206-Asp207-Asp311 coordinate Mg^{2+} ions and α-phosphate nucleotides, while His210-His233-Asp288 catalyze the hydrolyzation of triphosphates (Mauney and Hollis, 2018). Hydrolyzation of dNTPs may serve as an important and at present the only known mechanism of negative regulation of dNTP pool enrichment, critical to ensure genome stability and improve DNA synthesis fidelity (Franzolin et al., 2013). The importance of its role is supported by the evidence that an unbalanced dNTP pool may stimulate cell cycle arrest at the S-phase (Chabes and Stillman, 2007).

Therefore, SAMHD1 is involved in cell cycle in two ways: it is regulated by cell cycle proteins and impacts cell cycle progression, through dNTP pool control (Franzolin et al.,

2013). One of the first observations of a possible involvement of SAMHD1 in cell cycle regulation was the evidence that non-cycling $CD4^+$ T cells abundantly express SAMHD1 and are resistant to HIV-1 infection (Baldauf et al., 2012).

Later works revealed that SAMHD1 is active and negatively controls dNTPs during the G_0 phase, when cells are quiescent and they do not need dNTPs for DNA duplication (Sze et al., 2013). When cells exit from G_0 and enter in G_1 phase, SAMHD1 is phosphorylated at T592 and subsequently inactivated. This transition is facilitated by a mitogen-induced activation of Raf/Mek/Erk kinases and increase of cyclin A2/Cdk1 activity (Mlcochova et al., 2017) (**Figure 6a**). The $p21^{waf1/cip1}$ cell cycle inhibitor has been reported to influence SAMHD1 phosphorylation. In MDDCs growth *in vitro* with IFN-γ and CD40L, $p21^{waf1/cip1}$ increase of expression is associated with SAMHD1 dephosphorylation and dNTP pools decrease (Valle-Casuso et al., 2017) (**Figure 6b**). To support this observation, topoisomerase inhibitors-induced DNA damage results in activation of $p21^{waf1/cip1}$ and loss of SAMHD1 phosphorylation (Mlcochova et al., 2018). A recent work revealed that PP2A-B55α, a mammalian key mitotic exit phosphatase, can interact with and dephosphorylate SAMHD1 at T592 site in M/G_1 transition on actively proliferating cells (Schott et al., 2018) (**Figure 6c**). Moreover, it seems that SAMHD1 dynamic during cell cycle is finely regulated. Indeed, Tramentozzi and colleagues have recently shown that SAMHD1 is not simply activated/inactivated during the different phases of the cell cycle, but it is active and regulates dNTP pools during all cell cycle phases, controlling an excessive accumulation of DNA precursors even during the S-phase, when a too large amount of dNTPs could be detrimental for DNA replication fidelity (Tramentozzi et al., 2018).

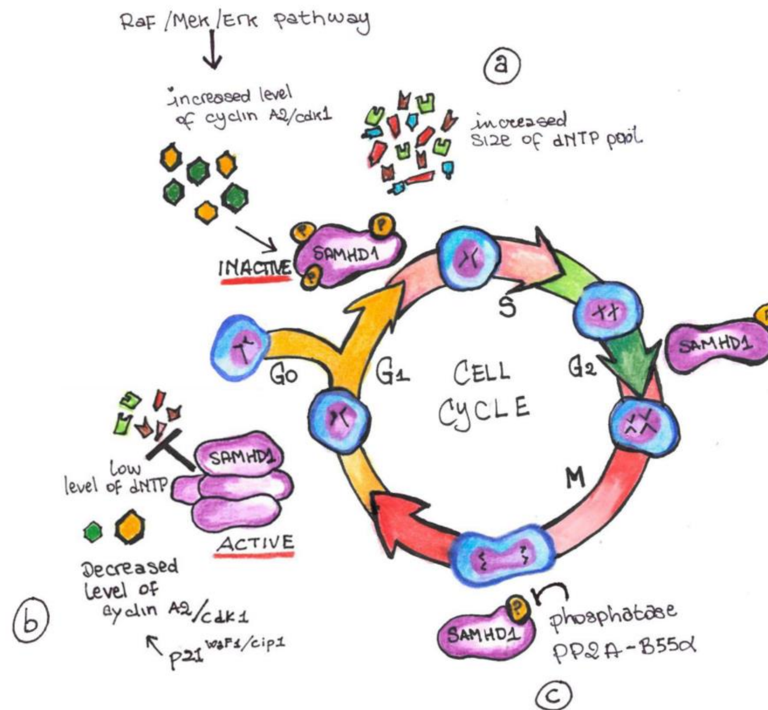

Figure 6: SAMHD1 expression and activation during cell cycle progression
SAMHD1 is highly expressed and active during G_0 phase but expressed at low level and inactive during S/G_2 phase. (a) Raf/Mek/Erk activation increases cyclin A2/Cdk1 activity, that in turn phosphorylate and inactivate SAMHD1 at the entry of the S phase, increasing intracellular dNTP levels. (b) IFN-α mediates the induction of $p21^{waf1/cip1}$ that decreases the levels of cyclin A2/Cdk1 and limits SAMHD1 phosphorylation. (c) PP2A-B55α phosphatase interacts with and dephosphorylates SAMHD1 at T592 site during M/G_1 transition (adapted from Sze et al., 2013)

In fact, during DNA replication, errors in the incorporation of new deoxynucleotides can physiologically occur. In steady state conditions, exogenous and endogenous stressors can create DNA damages, that can be detrimental for the cells and the entire organism; to limit the effect of these modifications, cells developed the so-called DNA damage responses (DDRs). When a break of the double helix of the DNA molecule occurs, the double strand break (DSB) can be repaired by two mechanisms: the non-homologous end-joining recombination (NHEJR), that directly ligates the two broken ends, and the homologous recombination (HR), that utilizes the homologous sequence of a sister chromatid to accurately repair the damage. SAMHD1 can be directly involved in HR mechanisms, recruiting the C-terminal binding protein interacting protein (CTIP) and the MRE11-RA50-NBS1 (MRN) nuclease complex (Daddacha et al., 2017) and acting as a scaffold protein for the "molecular actors" of HR. Notably, MRE11 is a 3'-5' exonuclease that

participates in resection of nucleotides at the stalled replication forks (Coquel et al., 2018), and it could contaminate SAMHD1 preparations for *in vitro* assays, and thus be responsible of the supposed, but controversial, exonuclease activity of SAMHD1 itself (Beloglazova et al., 2013; Seamon et al., 2015).

d. Mechanisms of regulation

SAMHD1 is active in a tetrameric form and the dynamic of oligomerization is complex and still under debate. One of the main features of tetramerization is the binding of dNTPs inside two allosteric sites, located at the interface between monomers, named A1 and A2 (Bhattacharya et al., 2016) (**Figure 7**). These two allosteric sites are located nearby: the A1 site binds specifically dGTP and GTP, while the A2 site is defined as a coactivator and it is more promiscuous, binding other dNTPs, despite it shows a preference for purines (Mauney and Hollis, 2018). Together, binding of dGTPs (or GTP) and dNTPs is fundamental for the formation of a long-lived tetrameric form. In their absence, SAMHD1 is inactive and monomers can only associate in a dimeric form, establishing a monomer-dimer equilibrium not enzymatically competent (Hansen et al., 2014).
The most characterized mechanism for formation and stabilization of active tetramer is phosphorylation of the SAMHD1 monomer at the C-terminal Threonine 592 (T592) residue. Human and murine SAMHD1 are substrates of phosphorylation at multiple sites, but T592 acquired, over time, a predominant importance for SAMHD1 activity and biology. The first observation of SAMHD1 phosphorylation at T592 was made by White and colleagues in 2013, when they identified a cyclin-dependent kinase target motif, ranging from amino acid 592 to 595 (^{592}TPQK^{595}), and described a differential phosphorylation status in cycling and noncycling THP-1 and CD4^{+} T cells. In non-cycling cells, SAMHD1 was unphosphorylated and active, and capable to restrict HIV-1 infection, while in cycling cells it became phosphorylated and unable to restrict HIV-1, despite the ability to hydrolyze dNTPs remained intact (White et al., 2013a).

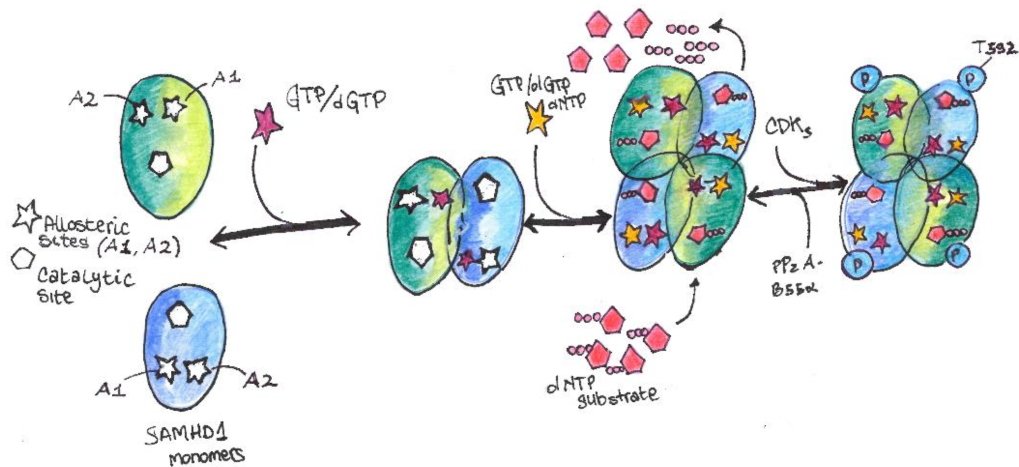

Figure 7: Model for SAMHD1 regulation of stabilization and activity
SAMHD1 activity is controlled by activator nucleotides, essential for preservation of nucleotide homeostasis. In low dNTPs condition, SAMHD1 is present in a monomer–dimer equilibrium. GTP binding in A1 stabilizes the dimer, while dNTPs binding at A2 favor SAMHD1 tetramerization and stabilization. Tetrameric SAMHD1 catalyzes dNTPs degradation and prevents their accumulation. T592 phosphorylation destabilizes tetramer stability apparently without modifying catalytic efficiency. PP2A-BB5α mediates SAMHD1 dephosphorylation (adapted from Mauney and Hollis, 2018).

In the same year, two other research groups pointed to the influence of T592 phosphorylation on the negative regulation of SAMHD1 antiviral activity, without the affection of dNTP pool regulation (Welbourn et al., 2013), and the involvement of cyclin A2/Cdk1 activity for an efficient phosphorylation (Cribier et al., 2013). Mass spectrometry analysis of human SAMHD1 conducted in 2014 by Gelais et al. confirmed the interaction with cyclin A2 and Cdk1 and revealed an interaction with Cdk2 and S-phase kinase-associated protein 2 (SKP2). All these proteins were expressed in cycling U937 and THP-1 monocytic cells, that are permissive to HIV-1 infection, suggesting a role of these proteins in negative regulation of SAMHD1 restrictive activity and establishing a cellular environment permissive to HIV-1 replication (Gelais et al., 2014). Despite at that time the precise effect of T592 phosphorylation on SAMHD1 function was unclear, later studies revealed that this post-translational modification prevented the long-lived and enzymatically active tetramer formation, and reported a correlation between the destabilization of the active tetrameric form by phosphorylation and the impairment of retroviral restriction, despite the dNTPase function seemed to be lowered but not completely abolished (Arnold et al., 2015; Yan et al., 2015). In summary, T592

phosphorylation inhibits SAMHD1 antiviral activity, despite dNTPase catalysis is not completely abolished, and it occurs through destabilization of the active homotetramer.

However, despite the extensive knowledge on SAMHD1 phosphorylation, little is known about its subsequent dephosphorylation. In a recent paper, it has been reported the only dephosphorylating enzyme known to date, the cellular serine/threonine protein phosphatase 2 A holoenzyme (PP2A), containing the regulatory subunit B55α (PP2A-B55α). The interaction with PP2A-B55α and SAMHD1 dephosphorylation has been characterized *in vitro* in non-cycling monocyte derived macrophages (MDMs), and it has been described to promote mitotic exit of the cells, through activation of SAMHD1 and subsequent reduction of dNTP pool at levels compatible with cellular quiescence (Schott et al., 2018).

In recent years, researchers focused on alternative mechanisms of SAMHD1 activity regulation, such as oxidation. This is a reversible post-translational modification that can be triggered by different stimuli and regulates the activity of proteins involved in cellular metabolism, cell signaling and cell cycle progression. SAMHD1 contains three important cysteines, Cys341, Cys350 and Cys522 that can be substrates of oxidation. When this happens, Cys341 and Cys350 can form a disulfide bond between themselves, and Mauney and collaborators proposed a model in which Cys522 act as a “switch” for protein oxidation, that causes conformational changes of allosteric sites, impairing dNTPs binding and destabilizing the formation of active tetramers (Mauney et al., 2017). Subsequently, Wang and colleagues reported that functional Cys341 and Cys522 are required for SAMHD1 restriction of HIV-1 infection, but that mutations in these sites did not affect dNTPs hydrolyzation and protein tetramerization. Given that, they proposed that oxidation of the protein is a key factor to discriminate dNTP metabolic activity and retroviral restriction (Wang et al., 2018).

Another mechanism proposed to regulate SAMHD1 activity is the acetylation at Lys405, a substrate for the acetyltransferase arrest-defective protein 1 (ARD1), which results in an increased dNTP hydrolyzing activity, during the G_1 phase of the cell cycle (Lee et al., 2017).

In summary, SAMHD1 phosphorylation, oxidation and acetylation may represent different means to control its catalytic activity and are a testimonial of the cellular need to precisely control and maintain the dNTP pool, in physiological conditions as well as in the context of viral infections.

e. Mutations

Given the central role of SAMHD1 in different metabolic and cellular pathways (i.e., dNTP metabolism, DNA damage response, innate immunity regulation and viral restriction), its mutations can be detrimental for the organism and can lead to specific diseases. One of the most characterized syndromes associated, among other genes, with SAMHD1 mutations is the Aicardi-Goutières syndrome (AGS). This is a type-I interferonopathy characterized by hyper IFN-I production and signaling. It is linked to altered nucleic acid metabolism and can cause the death of 40% of affected children (Crow and Rehwinkel, 2009; Crow et al., 2015). AGS can be caused by mutations of different genes beside SAMHD1 (i.e., TREX1, RNASEH2A, ADAR), all of them involved in nucleic acid metabolism. Thus, from a genetic point of view, it is a heterogeneous disease, but the common feature is the unusual accumulation of nucleic acids into the cell, the activation of sensing pathways and production of IFN-I (Crow and Rehwinkel, 2009). AGS related with SAMHD1 mutations can also cause cerebral vasculopathy and atherosclerosis, stroke and mitochondrial DNA aberrations (Leshinsky-Silver et al., 2011; Li et al., 2015; Xin et al., 2011).

SAMHD1 mutations also correlate with cancer development. Given the fact that a dysregulated quantity of dNTPs inside the cell can lead to genomic instability, mutations and cancer, recent researches pointed out that certain SAMHD1 mutations can be correlated with chronic lymphocytic leukaemia, T-cell prolymphocytic leukaemia, colon and lung cancers (Mauney and Hollis, 2018). For example, in colorectal cancer SAMHD1 heterozygous mutations affect negatively its triphosphohydrolase activity, with subsequent increase of dNTP pools and mutation rates, increasing the probability of cancer development (Rentoft et al., 2016). Most notably, Daddacha and colleagues reported that the SAMHD1 K484T mutation, that occurred in a patient affected by gastric cancer, retained its triphosphohydrolase activity, but showed an impaired homologous recombination (HR) mechanism (Daddacha et al., 2017).

4. SAMHD1 and viral restriction

a. HIV-1 restriction

From 2011 to 2013 were milestone years for research in SAMHD1 and its role in HIV-1 infection. In 2011, Hrecka and Laguette, in two independent research letters published in Nature, demonstrated SAMHD1 as the HIV-1 restriction factor counteracted by Vpx, an HIV-2/SIV encoded accessory protein responsible of SAMHD1 targeting for proteasomal degradation (Hrecka et al., 2011; Laguette et al., 2011). In fact, both research groups were interested in isolating and characterizing the factor responsible for the Vpx-mediated relieve of resistance to HIV-1 infection observed in dendritic cells and macrophages (Goujon et al., 2008), and both groups pointed at SAMHD1 as a key protein in this process. Vpx is not encoded by HIV-1, suggesting that overcoming of SAMHD1 activity is dispensable for this virus to ensure its spread in the organism. Interestingly, Kyei and collaborators hypothesized that the strategy to not degrade SAMHD1 is beneficial for HIV-1, because, unlike Vpx-encoding HIV-2/SIVmac viruses, HIV-1 cannot trigger cGAS-STING-mediated DNA sensing in macrophages, maybe due to a very low replication efficacy of viral genomes caused by SAMHD1 activity (Kyei et al., 2015). Thus, this strategy could be efficient to ensure the macrophages as an HIV-1 reservoir and player of viral transmission (Wei and Yu, 2015).

Notably, restriction of HIV-1 infection appears in MDM treated with IL-12 and IL-18 but not with M-CSF, and this observation correlated with an overexpression of SAMHD1 in IL-12/IL-18 treated MDM, leading to the conclusion that SAMHD1 is an effector of IL-12 and IL-18-mediated immune responses to HIV-1 infection and replication (Pauls et al., 2013). As stated above, the most accepted hypothesis that correlates SAMHD1 to HIV-1 restriction is the reduction of cellular dNTP pools necessary for viral genome replication. Despite this, many observations underlie the need to review this hypothesis, adding a yet undiscovered mechanism of HIV-1 restriction mediated by SAMHD1 and independent from its triphosphate hydrolase activity. In fact, one of the most important observations is that SAMHD1 phosphorylation impairs HIV-1 restriction but not dNTPase activity (Bhattacharya et al., 2016; Welbourn et al., 2013). For this reason, it was proposed that SAMHD1 can also degrade HIV-1 incoming RNA and prevent sensing of nucleic acids,

based on observations made in MDMs, CD4^{+} T cells and monocytes (Choi et al., 2015; Ryoo et al., 2014). However, these results are still under debate, since the hypothetic exonuclease activity of SAMHD1 has not been isolated yet, and it could derive from a contaminant in purified SAMHD1 preparations (Seamon et al., 2015), probably the exonuclease MRE11 that has been shown to bind SAMHD1 at replication forks (Coquel et al., 2018) (see also page 28).

b. SAMHD1 restriction of RNA and DNA viruses

Considering the importance that research on SAMHD1 involvement during HIV-1 infection acquired over time and the intense scientific debate that it has been able to stimulate, many research groups started to be interested on the eventuality that SAMHD1 could be a restriction factor against other viruses besides HIV-1, also because of its central role in dNTP pool and cell cycle regulation.
In a paper exploring the independent role of the two principal domains of SAMHD1 (SAM and HD domain), White and colleagues reported the involvement of SAMHD1 in restriction of other retroviruses. Intact SAMHD1 and the HD domain alone, expressed in PMA-treated U937 cells, were reported to potently inhibit Feline Immunodeficiency Virus (FIV), Equine Infectious Anaemia Virus (EIAV), Bovine Immunodeficiency Virus (BIV) and N-tropic/B-tropic Murine Leukaemia Virus (N-MLV; B-MLV) (**Table 3**). Notably, EIAV was inhibited by SAMHD1 to a lesser extent, suggesting a possible viral immune-evasion mechanism adopted by this virus (White et al., 2013).
Among RNA viruses, recent findings reported a role of SAMHD1 in the regulation of two important ssRNA+ viruses, Chikungunya and Zika, belonging to *Togaviridae* and *Flaviviridae* families, transmitted by *Aedes aegypti* and *Aedes albopictus* mosquitoes, respectively. HFFs infected with Chikungunya and Zika virus showed a significant up-regulation of proteins involved in IFN-I signalling pathway, including SAMHD1 (Wichit et al., 2019). Notably, Vpx-mediated SAMHD1 degradation inhibited Chikungunya and Zika virus replication, suggesting a counterintuitive and previously unreported pro-viral role of SAMHD1, as confirmed by increase in viral RNA levels and titers in SAMHD1 overexpressing U937 cells, normally poorly permissive for these viruses (Wichit et al.,

2019). Despite this, the exact SAMHD1-mediated pro-viral mechanism was not explored and remains unknown.

Table 3: SAMHD1 role in viral replication

Virus(es)	Classification	SAMHD1 role	Reference(s)
HIV-1	Retroviral ssRNA	Antiviral	Lahouassa et al., 2012
FIV, EIAV, BIV, N-MLV, B-MLV	Retroviral ssRNA	Antiviral	White et al., 2013
Chikungunya, Zika virus	ssRNA+	Proviral	Wichit et al., 2019
HSV-1	dsDNA	Antiviral	Kim et al., 2013 Hollenbaugh et al., 2013
VACV	Linear dsDNA	Antiviral	Hollenbaugh et al., 2013
HBV	Partially dsDNA	Antiviral	Chen et al., 2014
EBV	dsDNA	Antiviral	Zhang et al., 2019

The first observation of a role for SAMHD1 in non-retroviral infection restriction was made by Hollenbaugh and colleagues, who investigated the possible restriction of the dsDNA viruses Herpes simplex (HSV) and vaccinia (VACV) in myeloid cells (Hollenbaugh et al., 2013). They observed that Vpx-mediated SAMHD1 depletion in MDM infected with VACV led to increased concentrations of dNTPs, of a late gene expression (*A4* gene), and of viral particle production. Similar results were obtained in HSV-1 infection of THP-1 cells, where knocking-down SAMHD1 expression led to an increase of the HSV-1 encoded ICP-4 and UL27 proteins, and of viral particle production. Together, these results suggested that SAMHD1 can restrict infection also of dsDNA viruses in non-dividing myeloid cells (Hollenbaugh et al., 2013). The observation was confirmed in the same year by Kim and colleagues, who reported that SAMHD1 can inhibit HSV-1 replication, that T592 phosphorylation did not impact on SAMHD1 ability to restrict the virus and, most notably, that similarly to HIV-1, the restriction is exerted through depletion of dNTP pools (Kim et al., 2013).

Among DNA viruses, the impact of SAMHD1 on Hepatitis B Virus (HBV) replication is an area of scrutiny, probably due to the severity of symptoms and clinical outcomes caused by this virus. SAMHD1 is expressed by liver cells and restricts HBV replication in SMMC-7721 and BEL-7402 liver cells lines, apparently in a dNTP pool down-regulation independent mechanism (Chen et al., 2014). Moreover, HBV expressing HBx can downregulate SAMHD1 expression, compared to the HBx-defective virus. These results suggested that SAMHD1 can restrict HBV replication in a dNTP-independent mechanism, and that the virus can in turn overcome its antiviral activity (Chen et al., 2014). However, the exact mechanism of restriction is controversial, given the fact that later observations made on SAMHD1 mutants of catalytic D207 residue in the HD domain, reported an impaired capacity to reduce HBV replication, suggesting an essential role of the triphosphohydrolase activity (Jeong et al., 2016). Interestingly, recent data suggest that SAMHD1 can have different roles in different stages of HBV infection, in a yet undetermined mechanism. At early stages, it can promote the formation of covalently closed circular DNA (cccDNA) intermediates, that are essential for viral replication, but it can also negatively regulate reverse transcription, that occurs in later phases (Wing et al., 2019). Collectively, despite the debate on the exact restriction mechanism, SAMHD1 can promote or restrict HBV replication during different phases of infection.

In relation to other herpesviruses than HSV-1, during the writing of this thesis, two papers appeared in the same issue of Cell Reports, in July 2019, investigating the role of SAMHD1 in EBV (Zhang et al., 2019), and, with our surprise, in HCMV infection (Kim et al., 2019). EBV counteracted SAMHD1 activity by T592 phosphorylation mediated by viral BGLF-4 serine/threonine protein kinase, leading to inhibition of dNTPase activity. Moreover, it was shown that many conserved β- and γ-herpesvirus encoded kinases, including HCMV pUL97, can phosphorylate and inactivate SAMHD1 (Zhang et al., 2019). Notably, the paper published by Kim and colleagues on HCMV, confirmed the involvement of pUL97 in SAMHD1 phosphorylation (Kim et al., 2019). Given the similarity of our study, the findings published by these authors will be discussed in more details later.

Except for the paper by Kim and colleagues, to date there are no other information about the role of SAMHD1 in HCMV defence, and thus there are still many open questions in this field. Given the many points of connection between cellular mechanisms regulated by

SAMHD1 and involved in HCMV replication (e.g. cell cycle and dNTP pool regulation), together with the fact that the mechanisms beside the establishment of HCMV latent infection are still largely unknown, we questioned if SAMHD1 could have an effective role in limiting HCMV replication and viral particle production. We thus analysed the upregulation of SAMHD1 expression after infection in different cell types and with different viral strains, and we investigated possible mechanisms of viral intrinsic immune evasion. We then included in our analysis the cellular and viral proteins that are known to interact and phosphorylate SAMHD1, including pUL97, trying to deepen the molecular "fight" between the virus and the intracellular mechanisms of defence, and to reach new and unexplored observations that could help our understanding of HCMV-stimulated intrinsic antiviral-immune responses.

2. Aim of the study

The aim of this thesis work was to investigate the role of SAMHD1 in HCMV replication and potential viral evasion mechanisms, which could contribute to the unsuccessful clearance of the virus. Indeed, HCMV infection persists in more than a half of the human population, and the clinical features of the infectious disease can vary among individuals, depending on the fitness of their immune system. In fact, HCMV is an important opportunistic pathogen in immunocompromised hosts, like AIDS patients and transplant recipients, while it is asymptomatic in healthy individuals. Notably, latent infection is a key feature of the virus, which interferes with host antiviral responses, starting from innate immunity to adaptive immunity, and including host intrinsic restriction factors.

SAMHD1 is a cellular nuclear triphosphohydrolase (driving the conversion of deoxynucleotides triphosphates to deoxynucleosides and inorganic triphosphates), and so far, it is the only cellular protein known to negatively regulate dNTP pools. Moreover, it is a well-known retroviral restriction factor, particularly against HIV-1. Among DNA viruses, SAMHD1 is involved in Herpes Simplex Virus-1 (HSV-1), vaccinia virus and Hepatitis B Virus (HBV) restriction, while its role in HCMV infection is now emerging. SAMHD1 functions are tightly intertwined between cell cycle regulation, viral replication restriction and regulation of deoxynucleotides metabolism. It acts in an active tetrameric form and it is mainly regulated by Thr-592 phosphorylation (P-T592), that destabilizes tetramerization and restriction activity.

In this work, we investigated mRNA and protein expression, localization and post-translational modifications of human SAMHD1 after infection of different cell types with different HCMV strains. We analysed the contribution of SAMHD1 phosphorylation at T592 residue on HCMV replication, by overexpression of SAMHD1 mutants at this site and by using chemical inhibitors of known cellular and viral kinases, potentially involved in SAMHD1 phosphorylation, to unveil its potential role in viral restriction. Moreover, we analysed intracellular SAMHD1 localization and interaction with viral structures and proteins, aiming at characterizing possible immune evasion mechanisms.

Our results show the increase of SAMHD1 expression and phosphorylation upon HCMV infection, and the involvement of cellular but not viral kinases in its phosphorylation. SAMHD1 does not seem to restrict HCMV replication, probably due to delocalization of

phospho-SAMHD1 in the cytoplasm, and its interaction with HCMV infectious particles and non-infectious dense bodies. Moreover, we show preliminary data of HCMV proteins interacting with SAMHD1, that could explain the observed cytoplasmic localization upon infection and the lack of restrictive activity. Altogether, our study suggests an HCMV countermeasure targeting SAMHD1, that might potentiate viral replication and contribute to the success of viral spread inside the host.

3. Materials and methods

1. Cells and culture conditions

Primary human foreskin fibroblasts (HFFs), human embryo kidney 293T (HEK 293T) and adult retinal pigment epithelial cell line-19 (ARPE-19) were purchased from ATCC. HFFs and HEK 293T cells were grown in Dulbecco's modified eagle medium (DMEM) containing 10% FCS, glutamine, sodium pyruvate, 100 U/ml penicillin and 100 ug/ml streptomycin sulfate. HFFs were used at passages comprised from 10 to 20.
ARPE-19 cells were grown in a 1:1 mixture of DMEM and Ham's F-12 medium (Invitrogen) containing 10% FCS, 15 mM hepes, glutamine, sodium pyruvate, 100 U/ml penicillin and 100 ug/ml streptomycin sulfate.
Human dermal microvascular endothelial cells (HMVECs) were obtained from Clonetics (San Diego, CA) and cultured in endothelial growth medium (EGM) containing 10% FCS, human recombinant vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), human epidermal growth factor (hEGF), insulin growth factor (IGF-1), hydrocortisone, ascorbic acid and heparin. Cells were seeded into culture dishes coated with 0.2% gelatin. Experiments were carried out with cells at passages 4 to 15. All cells were maintained at 37° C in a 5% CO_2 atmosphere.

2. HCMV preparation and infection

HCMV AD169 strain (ATCC VR538) was prepared by infecting 80-90% confluent low passage HFFs at multiplicity of infection (MOI) 0.01 PFU/ml. After 3 hours post infection (h.p.i.) at 37°C, the virus was removed and replaced with fresh medium (DMEM 10% FCS). The cells were cultured until a marked cytopathic effect was seen. Stocks were prepared after three rounds of cell freezing and thawing, subjected to centrifugal clarification, aliquoted and frozen at -80°C. Virus titer was measured by standard plaque assay on HFFs. The concentration used in the experiments was 2.0 x 10^7 PFU/ml.
HCMV TR was derived from an ocular specimen (Smith et al., 1998), and after a few passages on fibroblasts, was cloned into a bacterial artificial chromosome (BAC) (Murphy et al., 2003; Ryckman et al., 2006). Reconstitution of infectious TR was performed as previously described (Bronzini et al., 2012) by co-transfecting HFFs with the

corresponding TR-BAC and a plasmid expressing HCMV pp71. Reconstituted infectious virus retained the ability to infect endothelial and epithelial cells, as well as monocytes and macrophages (Bronzini et al., 2012; Ryckman et al., 2006). HCMV VR-1814 is a derivative of a clinical isolate recovered from a cervical swab of a pregnant woman (Grazia Revello et al., 2001). This strain was propagated in HUVEC and titrated as previously described (Caposio et al., 2007).

Cells were infected at 80-90% confluence at MOI 1 or MOI 0.05, in their respective culture medium, without FCS. After 3 h.p.i (AD169 and TR strains) or 24 h.p.i (VR-1814 strain) at 37°C, virus inoculum was replaced with fresh culture medium. Mock-infected cells were treated with the same amount of culture medium and for the same time lapse.

When kinase inhibitors were used, HFFs were pre-treated with the specific inhibitor, then infected in the presence of the inhibitor at the indicated concentration. After the virus inoculum was removed, the same drugs were added again at the same concentration. Cells treated with DMSO or not treated (nt) were used as experimental controls.

3. Real-time PCR

Total RNA was extracted using TRI Reagent solution (Life Technologies), according to the manufacturer's instructions and 1 µg of total RNA was used for cDNA synthesis in a reaction volume of 20 µl. Real-time PCR was performed using the ABI Prism 7000 PCR cycler sequence detection system (Applied Biosystems, Foster City, CA). cDNAs were amplified with primers for SAMHD1 (Hs.PT.49a.21502281) and GAPDH (Hs.PT.49a.2918858) using specific TaqMan gene expression assays (Integrated DNA Technologies, Coralville, IA). Relative expression of SAMHD1 gene versus GAPDH was calculated with the $2^{-\Delta\Delta Ct}$ method.

4. Antibodies for immunoblotting and flow cytometry

Rabbit polyclonal antibodies specific for phosphorylated SAMHD1 at residue T592 (8005), total SAMHD1 protein (12586-1-AP), and the p85 subunit of PI3K, N-SH2 domain (#ABS233) were purchased from ProSci (Poway, CA), Proteintech (Manchester, UK) and Millipore (Temecula, CA), respectively. Anti-phospho-SAMHD1 and anti-SAMHD1 antibodies were diluted 1:1000 in Tris buffered saline containing 0,02% Tween-

20 and 1% BSA (TBST). Anti-p85 antibody was diluted 1:2000 in TBST with 3% BSA. Mouse monoclonal antibody specific for IE1/IE2 viral proteins (MAB810R) was purchased from Millipore and diluted 1:1000 in TBST with 5% milk. Horseradish peroxidase-conjugated (HRP-conjugated) secondary anti-rabbit and anti-mouse antibodies were purchased from GE Healthcare (Pittsburgh, PA). All these antibodies were used in immunoblotting experiments.

5. Immunoblot analysis

Cells were lysed for 20 min at 4°C in a lysis buffer containing 0.2% Triton X-100, 0.3% Nonidet P-40 (NP-40), 1 mM EDTA, 50 mM TrisHCl pH 7.6 and 150 mM NaCl, protease and phosphatase inhibitors to obtain whole-cell protein extracts. Protein concentration was measured with the Bio-Rad protein assay (BPA) (Bio-Rad) at the Victor 2_{TM} 1420 multilabel counter system (Perkin Elmer). 20-40 µg of total cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amersham, GE Healthcare). Membranes were blocked with TBST with 5% milk blocking buffer and probed with the indicated antibodies. Immunoreactivity was revealed using an enhanced chemiluminescence (ECL) kit (Amersham, GE Healthcare). Densitometric analysis was performed with ImageJ software.

6. Immunofluorescence and FACS analysis

Uninfected and infected cells were harvested at the indicated time post-infection. For intracellular staining of IE1/IE2 antigens, cells were fixed in PBS methanol-free 1% formaldehyde, permeabilized with 70% cold ethanol and then incubated with alexa fluor 488-conjugated anti-IE1/IE2 mAb (Mab810X) (Millipore). Cells were acquired with a FACS Calibur (BD Biosciences) flow cytometer and analyzed with FlowJo 10 software (ver 10.0.7).

7. Chemical compounds

The chemical compounds used were the selective inhibitor of Cdk1 CGP74514A (CGP; Millipore), the inhibitors of pUL97 HCMV kinase Maribavir (MBV; kindly provided by Dr. Valentina Dell'Oste) and Gö6976 (Calbiochem). All these compounds were diluted in dimethyl sulfoxide (DMSO; Sigma-Aldrich). Other reagents were methylcellulose (Methocel MC), gelatin, CHAPS, octyl-β-D-glucopyranoside and crystal violet, all from Sigma-Aldrich (St. Louis, MO), lipofectamine 2000 from Invitrogen (Thermo Fisher Scientific, Waltham, MA).

8. Small interfering RNA

Small interfering RNA (siRNA) specific for SAMHD1 (sc-76442) was obtained from Santa Cruz Biotechnology. Non-targeting siRNA (siCtrl) was from Dharmacon (Lafayette, CO). HFFs (90% confluence) were transfected with 200-300 nM siRNA using DharmaFECT siRNA transfection reagent (Thermo Fisher Scientific), according to the manufacturer's recommendation. 3 days after transfection, cells were infected with AD169 at MOI 1 or MOI 0.05. Cells and supernatants were harvested and analyzed respectively at 72 h.p.i and 6 d.p.i.

9. VLP generation

For VLP production, $3.5x10^6$ 293T cells were transiently transfected with SIV-based packaging plasmid with or without Vpx protein (pAdSiv3+ or pAdSiv3+/ΔVpx respectively) and VSV.G plasmid, in a ratio 2:1 by Calcium Phosphate method, using the Profection Mammalian Transfection System (Promega Corporation, Madison, WI, USA). 48 and 72 h post-transfection, cell culture supernatants were collected, cleared from cellular debris by low-speed centrifugation and passed through a 0.45-µm pore size filter (Millipore Corporation, Billerica, MA, USA). VLPs stocks were titred by the reverse transcriptase (RT) activity ($1.1x10^6$ cpm/ml). For HFF challenge, VLPs at MOI 1 were adsorbed by spinoculation at 1500 rpm for 30 min at room temperature, then incubated for 2 h at 37° C in a 5% CO2 atmosphere, and soon after the same cells were infected with

AD169 at a MOI of 1. At different days post-infection, cells and supernatants were harvested and subjected to immunoblotting and plaque assays, respectively.

10. Retroviral vectors production and infection

pOz plasmids containing gene encoding SAMHD1 wild-type, T592A or T592E mutants were kindly provided by Drs. M. Benkirane and A. Cribier (University of Montpellier, France). For retrovirus production, the Phoenix retrovirus packaging cell line 293T was transfected with pOZ plasmids and the packaging vectors (pCMV gag-pol and pMD2.G) using Lipofectamine 2000 (Invitrogen, San Diego). After 48 h, virus-containing supernatants were harvested, filtered, and stored at -80°C. Two infection cycles were then performed in 6-well plates on 90% confluent HFFs, with 1 ml/cycle of viral supernatant containing Polybrene. Supernatants were then replaced with fresh DMEM 10% FCS. After 3 days, cells were infected with AD169 at a MOI 1 or MOI 0.05, and finally harvested at 3 or 6 d.p.i, respectively.

11. Confocal microscopy analysis

HFFs were plated in 24-well plates and starved at 0.2% FCS. After 48 h, cells were infected with AD169 at MOI 1 for 3 h at 37°C. 48 h after infection, cells were plated on PBS 2% gelatin-coated multichamber, leaved over-night at 37°C and then fixed with PBS 4% paraformaldehyde, glycine 0.1 M and permeabilized with PBS 0.1% Triton X-100. After permeabilization, cells were blocked with PBS with 0.01% Triton X-100 and 1% CMV- human serum (obtained from a CMV seronegative donor) blocking buffer and incubated with the primary antibodies. After washes, cells were incubated with anti-rabbit (alexa fluor 594) and anti-mouse (alexa fluor 488) secondary antibodies. After 1 h of incubation, cells were washed and counterstained with DAPI (Life Technologies, D1306). Coverslips were mounted using slow fade gold reagent (Life Technologies, S36936). Images were acquired with IX83 FV1200 MPE laser-scanning confocal microscope with a 60 × /1.35 NA UPlanSAPO oil immersion objective. Images were processed with Fiji ImageJ software.

12. Nuclear/cytoplasmic fractionation

Cells were differentially lysed for cytoplasmic and nuclear protein extraction. Cytosolic proteins were obtained by incubating cell pellets for 4 min on ice in a hypotonic lysis buffer containing 50 mM KCl, 0.5% NP-40, 25 mM hepes pH 7.8, DTT 100 µM, protease and phosphatase inhibitors. Lysates were then centrifuged at 5000 rpm for 5 min, and supernatants containing cytoplasmic content stored at -80°C. The remaining nuclear pellets were then washed with a wash buffer containing 50 mM KCl, 25 mM hepes pH 7.8, protease and phosphatase inhibitors, and then lysed in the nuclear extraction buffer containing 500 mM KCl, 25 mM hepes pH 7.8, 10% glycerol, protease and phosphatase inhibitors. After vortexing, lysates were put on rotation for 2 h at +4°C and then stored at -80°C. Lysates were then immunoblotted as described above. Lamin-A and α-tubulin were stained to validate fractionation efficiency.

13. Cryo-immunoelectron microscopy

Cells grown in monolayer were fixed in 2% PFA/0.2% glutaraldehyde in 0.1 M PBS, pH 7.4, for 3 h at room temperature, then embedded into 12% gelatin (Sigma-Aldrich) in 0.1 M PBS, pH 7.4, solidified on ice, infused in 2.3 M sucrose overnight at 4°C, mounted on aluminum pins and frozen in liquid nitrogen. Ultrathin cryosections (~ 60 nm) collected with 1% methylcellulose in 1.15 M sucrose were immunolabeled with primary antibodies to SAMHD1 (Proteintech) and phospho-SAMHD1 (ProSci). Bound antibodies were visualized using goat anti-rabbit conjugated with 5-nm (Sigma) or 10-nm gold particles (Cytodiagnostics). Cryosections were analyzed with a Philips CM10 TEM.

14. Immunoprecipitation and mass spectrometry

Cells were lysed in a lysis buffer containing 50 mM Tris HCl pH 7.6, 150 mM NaCl, 1mM EDTA, 1% octyl-β-D-glucopyranoside (Sigma-Aldrich), protease and phosphatase inhibitors and then pre-cleared with Protein-A-sepharose, for 1 h at 4°C. In parallel, Protein-A/sepharose was incubated with anti-SAMHD1 antibody, or control rabbit IgG in incubation buffer containing 50 mM Tris HCl pH 7.6, 150 mM NaCl, 5mM EDTA, 0.25 % NP-40 and 2% BSA, for 2 h at 4°C. The pre-cleared lysates were then incubated with

Protein-A-sepharose/antibody complexes for 2 h at 4°C. The immunoprecipitated samples were washed in washing buffer containing 50 mM Tris HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 0.1 % NP-40 and then resolved for 15’ in a 4-20% gradient SDS-PAGE. After this, gels were incubated in a fixing solution containing 25% isopropanol and 10% acetic acid, and then stained for 10 min at room temperature with a Coomassie blue solution containing 10% acetic acid and 0.006% Coomassie brilliant blue G-250 (Sigma). Gels were then de-stained over-night at 4°C in a solution containing 10% acetic acid in water. Gels were then cutted in small pieces (1 cm) and then processed for mass spectrometry analysis at the Division of System Biology at Science for Life Laboratory (Solna, Sweden).

15. Statistical analysis

Data analysis were performed using a paired Student *t* test. A p value < 0.05 was considered significant.

4. Results

1. SAMHD1 and APOBEC3s expression are up-regulated following HCMV infection

SAMHD1 and the family of APOBEC3 proteins are involved in restriction of many RNA and DNA viruses, comprising HIV-1, HBV and HSV-1 (Chemudupati et al., 2019). To test the hypothesis that HCMV could induce an intrinsic immune response mediated by these restriction factors, we infected human primary foreskin fibroblasts (HFFs) with various HCMV strains at a MOI of 1 and 5 and we evaluated APOBEC3 and SAMHD1 mRNA expression levels at different hours post-infection (h.p.i) by real-time PCR (**Figure 8**).

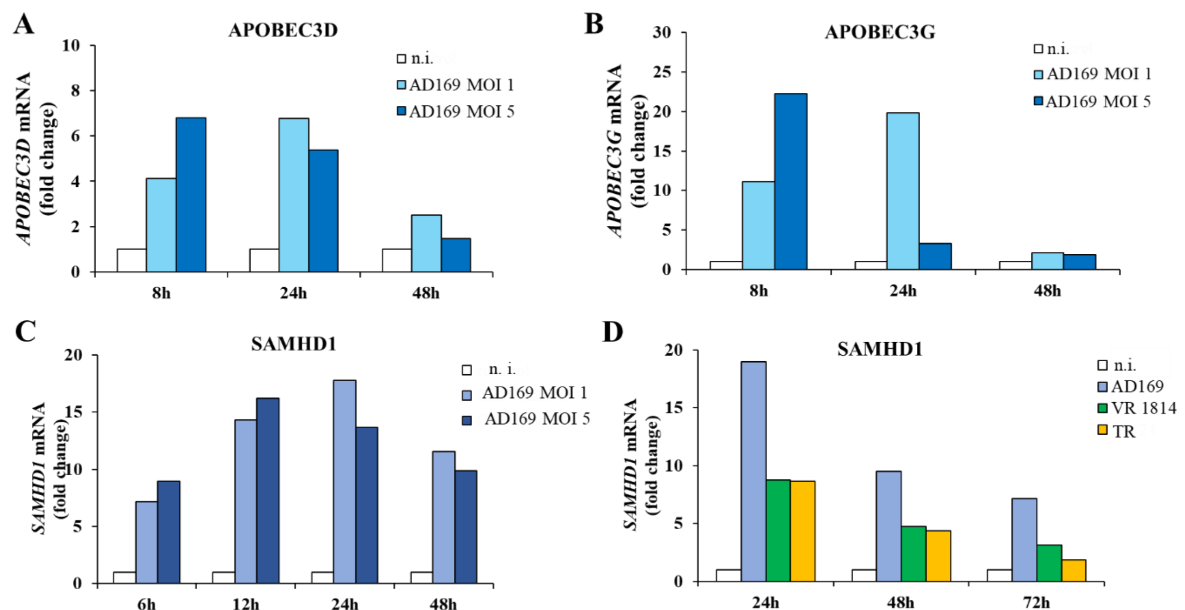

Figure 8. mRNA expression levels of APOBEC3D/G and SAMHD1 upon HCMV infection
Primary human foreskin fibroblasts (HFFs) were infected with HCMV AD169 (A-B-C) at a MOI of 1 and 5, or VR-1814 and TR strains (D) at a MOI of 1, or not infected (n.i.). At the indicated times post-infection, real-time PCR was performed using specific primers for *APOBEC3D, APOBEC3G*, *SAMHD1,* or for the *GAPDH* housekeeping gene. Data from one representative experiment out of 3 were normalized with GAPDH and referred to n.i. cells considered as calibrators and set at 1.

HCMV infection of HFFs with the laboratory strain AD169 led to an increase of APOBEC3D/G (**Figure 8A-B**) and SAMHD1 (**Figure 8C-D**) mRNA levels compared to uninfected cells. The increase was already detectable at 6-8 h.p.i, reached its peak at 24

h.p.i and decreased at 48 h.p.i. We evaluated also APOBEC3C/H/B mRNA levels, but their increase in infected cells was very low compared to APOBEC3D and APOBEC3G (data not shown).

When the project started, the APOBEC3 protein family was already extensively studied in limiting the replication of many viruses (Stavrou and Ross, 2015). On the contrary, SAMHD1 was mostly studied as an HIV-1 restriction factor. Therefore, we choose to explore its possible involvement in HCMV restriction, since there were no published data. We thus extended the analysis of SAMHD1 mRNA levels, in HFF infected also with the clinical strains VR-1814 and TR, and we observed an increase with all the strain tested (**Figure 8D**).

Next, to test the hypothesis that the increase of mRNA expression levels could result in an increase of SAMHD1 protein levels, we performed a kinetic experiment, by infecting cells with AD169 from 8 hours to 7 days. Immunoblotting analysis showed that SAMHD1 protein was detectable in uninfected cells, and its expression increased already at 24 h.p.i, reached its peak between 48 and 72 h.p.i and decreased at later times post-infection (**Figure 9A**). The time of 72 h.p.i was then chosen for the following experiments as a standard time for analysis, because the virus requires around 48-72 hours to reach the stage of complete maturation and progeny release (Mocarski et al., 2013). Densitometric analysis of SAMHD1 expression at 72 h.p.i. revealed a ~2-fold increase, compared to uninfected cells (**Figure 9B**).

We then questioned if the increase of protein expression was strain-dependent. To test this hypothesis, we infected HFFs with VR-1814 and TR clinical isolates and observed that the increase of SAMHD1 protein expression occurred with all the strains tested, with a major effect caused by VR-1814 and AD169 (**Figure 9C-D**). Moreover, the same trend was observed also in ARPE-19 (epithelial) and HMVEC (endothelial) cells and with all the strains tested (data not shown). All together these results showed that SAMHD1 mRNA and protein levels increase after HCMV infection and that this effect is strain- and cell-type independent.

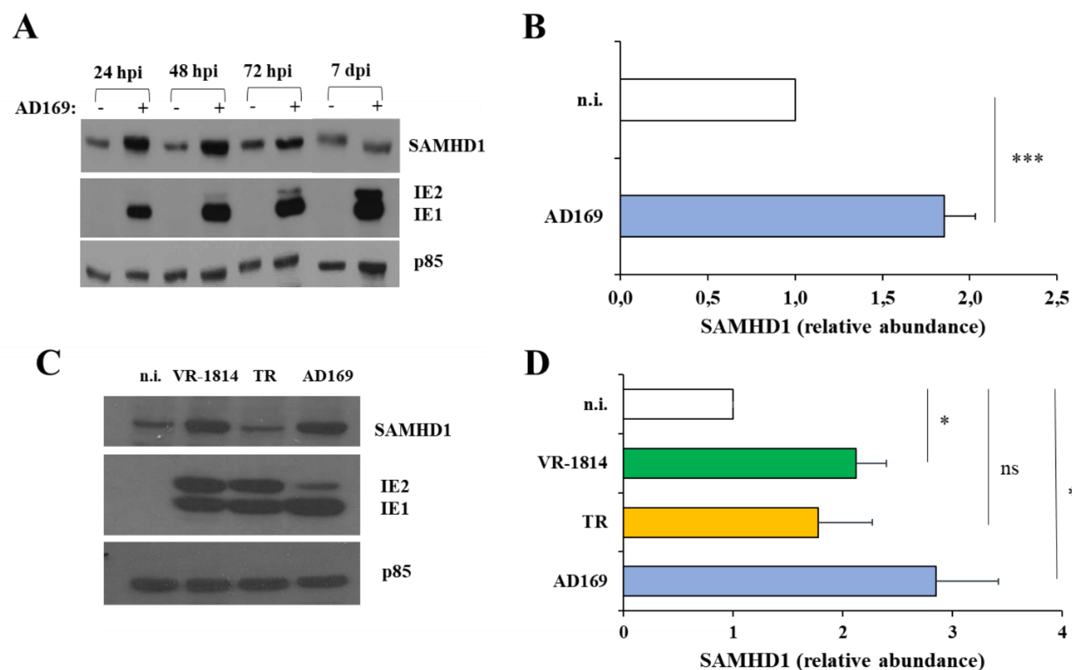

Figure 9. SAMHD1 protein expression levels upon HCMV infection

A) HFFs were not infected (-) or infected (+) with AD169 at a MOI of 1. At the indicated times post-infection, immunoblot of total cell lysates was performed using primary antibodies specific for the indicated proteins. Expression of IE1/IE2 viral antigens was used as control for infection, while the p85 subunit of PI3K was used as loading control. B) The relative abundance of SAMHD1 protein at 72 h.p.i, normalized to that of p85, was determined by densitometric analysis and is relative to SAMHD1 expression in uninfected (n.i.) cells, which was arbitrarily set as 1. Data are expressed as mean, and error bars represent the standard errors calculated from 7 independent experiments. C) Expression of SAMHD1 in HFFs infected with the indicated HCMV strains at a MOI of 1 at 72 h.p.i from one representative experiment out of 2 is shown. D) The relative amount of SAMHD1 protein in HFFs infected with the indicated strains was determined as in panel B. Error bars represent the standard errors calculated from 5 independent experiments. *p* values were calculated by Student *t* test. * $p < 0.05$; *** $p < 0.001$; ns, not significant.

2. SAMHD1 silencing and Vpx-mediated knocking-down marginally influence HCMV replication

Since AD169 led to a more robust increase of both mRNA and protein expression, we focused on SAMHD1 responses after AD169 infection in HFF cells.

To test the hypothesis that SAMHD1 could have a role in limiting HCMV replication, we transiently transfected HFFs with siRNA targeting SAMHD1 mRNA, or with a non-targeting siRNA. Three days after transfection, we infected HFFs with AD169 and, at 72 h.p.i., we evaluated the amount of SAMHD1 protein by immunoblot and immunofluorescence, the percentage of IE^+ cells by intracellular flow cytometry, and the level of viral production by standard plaque assay (**Figure 10**).

Immunoblot and immunofluorescence analysis confirmed the reduction of SAMHD1 protein levels with siRNA targeting SAMHD1 compared to scramble control (**Figure 10A-C**), and densitometric analysis showed that the inhibition of SAMHD1 expression in infected cells was of ~ 70%, compared to infected scramble control (**Figure 10B**). However, the production of complete HCMV infectious virions showed only a slight increase compared to scramble control (**Figure 10D**), while the percentage of IE^{+} cells, measured by FACS analysis, did not vary between siRNAs (data not shown). Experiments conducted at a low MOI (0,05) and analyzed at 6 d.p.i. showed similar results, indicating that the lack of a significant impact of SAMHD1 on HCMV replication is independent from the strength of the infection (data not shown).

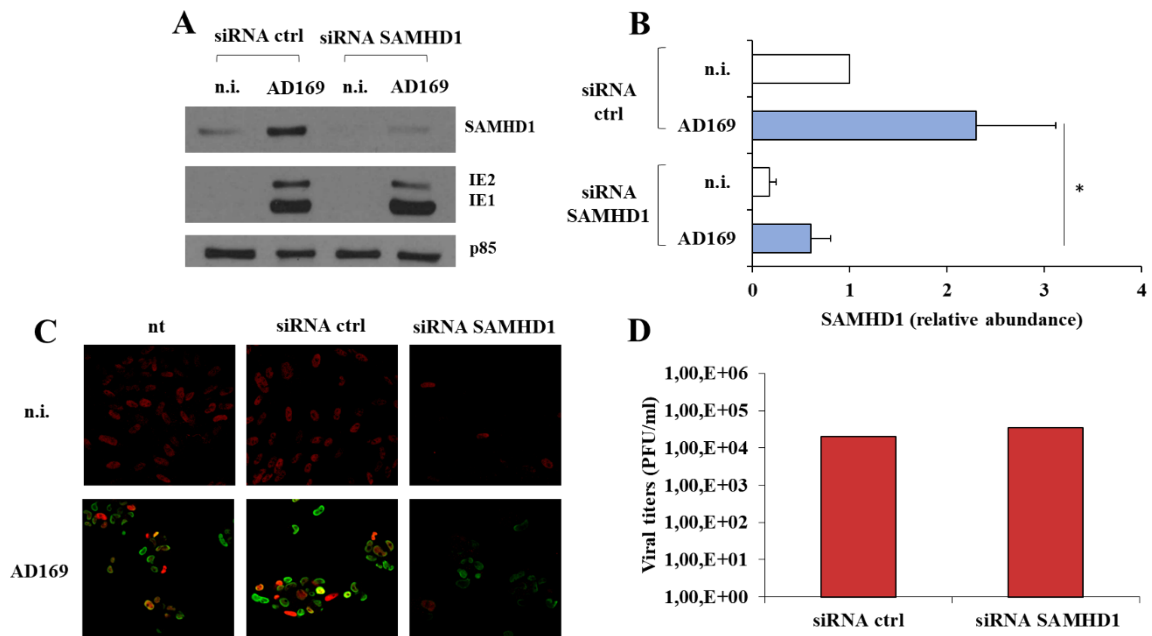

Figure 10. Influence of SAMHD1 silencing on HCMV replication
HFFs were transiently transfected with scramble siRNA (siRNA ctrl) or with siRNA targeting SAMHD1. Three days after transfection, cells were infected at a MOI of 1 and then harvested at 72 h.p.i. A) Levels of SAMHD1 and IE1/IE2 viral proteins expression were evaluated by immunoblot. The p85 protein was used as loading control. One representative experiment out of 5 is shown. B) The relative abundance of SAMHD1 protein in infected cells, normalized to p85 expression, was determined by densitometric analysis and it is relative to that of n.i. cells treated with a scramble siRNA (siRNA ctrl), arbitrarily set as 1. Data are expressed as mean and error bars represent the standard errors calculated from 5 independent experiments. *p* values were calculated by a Student *t* test. * $p < 0.05$. C) SAMHD1 expression was evaluated on the same cells by immunofluorescence. Red: SAMHD1; Green: IE1/IE2. D) Cell culture supernatants were assayed for infectious virus production by standard plaque assays. One representative experiment out of 5 is shown.

HIV-2-encoded Vpx has been reported to bind SAMHD1, induce its proteasomal degradation and thus relieve HIV-1 restriction (Laguette et al., 2011). After this finding, Vpx-mediated depletion of SAMHD1 became an important tool for the study of the involvement of this RF in viral control (Hollenbaugh et al., 2013; Wichit et al., 2019). Therefore, alternatively to the siRNA approach, we infected HFFs with SIV viral-like particles (VLPs) loaded with Vpx, and then we infected them with AD169 at MOI of 1. SAMHD1 expression levels and viral titers in the supernatant of infected cells where evaluated at different times post-infection by immunoblot and plaque assay, respectively (**Figure 11**).

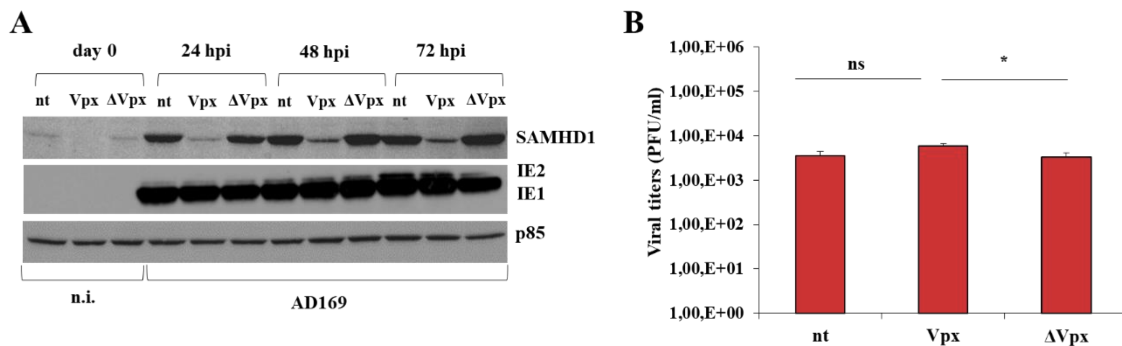

Figure 11. Influence of Vpx-mediated knocking-down of SAMHD1 expression on HCMV replication
HFFs were infected with VLPs loaded with Vpx, or not (ΔVpx) at a MOI of 1, and after 2 hours the cells were infected with AD169 at a MOI of 1. At different hours post-infection, cells and supernatants were harvested and subjected to immunoblot analysis and plaque assay, respectively. A) SAMHD1 protein expression levels were assayed by immunoblot. IE1/IE2 was used as control of infection, and p85 as the loading control. One representative experiment out of 5 is shown. B) Cell culture supernatants were assayed for infectious virus production by standard plaque assay. Data are expressed as mean and error bars represent the standard errors calculated from 5 independent experiments at 72 h.p.i. p values were calculated by a Student t test. * $p < 0.05$; ns, not significant.

Treatment with VLPs loaded with Vpx led to a dramatic decrease of SAMHD1 protein levels, compared to Vpx-deficient VLPs (ΔVpx) or not treated cells (**Figure 11A**). Despite the drastic reduction of SAMHD1 expression, the efficacy of replication of HCMV at 72 h.p.i. showed only a slight increase of approximately 2-fold (**Figure 11B**).
Altogether, these results suggest that SAMHD1 knock-down marginally influences HCMV replication.

3. SAMHD1 is phosphorylated at Threonine 592 in HCMV infected cells

SAMHD1 is negatively regulated by phosphorylation in the regulatory site T592, causing an impairment of restriction activity (White et al., 2013a). Indeed, cycling CD4^{+} T cells, that contains high levels of phosphorylated SAMHD1, are highly permissive to HIV-1 infection, compared to resting CD4^{+} T cells, where SAMHD1 in unphosphorylated (Cribier et al., 2013). To test the hypothesis that SAMHD1 was phosphorylated also in HCMV-infected cells and this could influence HCMV replication, we infected HFFs with laboratory and clinical strains and at 72 h.p.i we analyzed SAMHD1 T592 phosphorylation status by immunoblot with a specific phospho-T592 antibody (**Figure 12**).

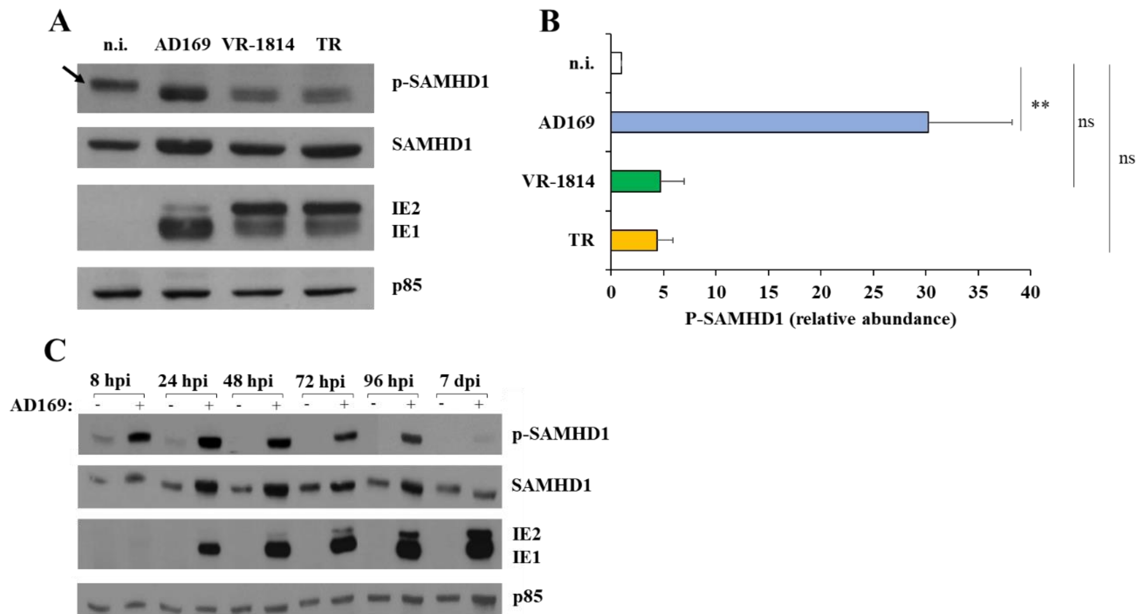

Figure 12. SAMHD1 T592 phosphorylation upon HCMV infection

A) HFFs not infected (n.i.) or infected with AD169, VR-1814 and TR strains at a MOI of 1 were lysed and analyzed for SAMHD1 T592 phosphorylation by immunoblot at 72 h.p.i. IE1/IE2 was used as control of infection, and p85 as loading control. One representative experiment out of 3 is shown. Arrow: unspecific band. B) The relative abundance of phospho-T592 SAMHD1, normalized to p85 expression, was determined by densitometric analysis and it is relative to that of n.i. cells, arbitrarily set as 1. Data are expressed as mean and error bars represent the standard error calculated from 3 independent experiments for VR-1814 and TR, and from 10 independent experiments for AD169. *p* values were calculated by a Student *t* test. ** $p < 0.01$; ns, not significant.

While in uninfected cells phospho-T592 SAMHD1 was undetectable, we observed that SAMHD1 was phosphorylated in cells infected with all the strains tested, though the AD169 laboratory strain led to a more prominent induction of phosphorylation, compared

to VR-1814 and TR (**Figure 12A**). In fact, densitometric analysis showed a 30-fold increase with AD169, compared to uninfected cells (**Figure 12B**). Moreover, immunoblot analysis performed at different times post-infection revealed that SAMHD1 phosphorylation was detectable already at 8 h.p.i and up to 96 h.p.i, and declined afterwards (**Figure 12C**).

All together, these results show that SAMHD1 T592 phosphorylation occurs in HCMV-infected cells in a strain-independent manner and from early times post-infection.

4. The viral pUL97 kinase inhibitor Maribavir reduces HCMV replication but has only a slight effect on SAMHD1 phosphorylation

The HCMV-encoded kinase pUL97 is one of the most important protein kinases encoded by the virus and it is critical for viral DNA replication (Prichard, 2009). It is a serine-threonine kinase expressed at 8-16 h.p.i. and it phosphorylates many viral and cellular proteins (Gill et al., 2012; Michel et al., 1996). Among its targets, pUL97 has been described to phosphorylate the restriction factor IFI16 to evade its antiviral function (Dell'Oste et al., 2014). We thus hypothesized that the early protein pUL97 may have a role in SAMHD1 phosphorylation, since T592 residue seems to be phosphorylated at early times post-infection (**Figure 12C**), probably resulting in a limited antiviral restriction capacity. To test this hypothesis, we used two chemical inhibitors targeting pUL97. Maribavir (MBV; 1263W94) is a potent antiviral drug that targets pUL97 (Biron et al., 2002) and it was extensively used to study pUL97 functions (Chou, 2008). In addition, indolocarbazole compound Gö6976, has been described to inhibit pUL97 kinase activity as well (Marschall et al., 2011). Thus, we first infected HFFs with AD169 at two different MOI, in the presence of MBV at 2 or 20 μM, or of DMSO as control. At different times post-infection, we evaluated the levels of SAMHD1 T592 phosphorylation by immunoblot, the percentage of IE^+ cells by intracellular flow cytometry, and viral titers in cell culture supernatants by plaque assay (**Figure 13**).

MBV treatment did not affect SAMHD1 T592 phosphorylation at 24 h.p.i (**Figure 13A**), as well as at times post-infection ranging from 8 hours to 6 days (data not shown). Compared to DMSO control, on infected cells at MOI 1 and MOI 0.05, MBV at 2 μM reduced HCMV replication respectively of ~35 fold and ~10 fold, while at 20 μM HCMV replication was reduced respectively of ~125 fold and ~20 fold, as assessed at 72 h.p.i

(**Figure 13B**). Notably, the percentage of IE$^+$ cells was not influenced by either treatment (**Figure 13C**). Moreover, similarly to MBV, Gö6976 treatment did not affect SAMHD1 phosphorylation, as shown at 72 h.p.i (**Figure 13D**).

All together, these results suggest that the HCMV kinase pUL97 does not play a major role in SAMHD1 phosphorylation, and that the MBV-mediated inhibition of HCMV replication is SAMHD1-independent.

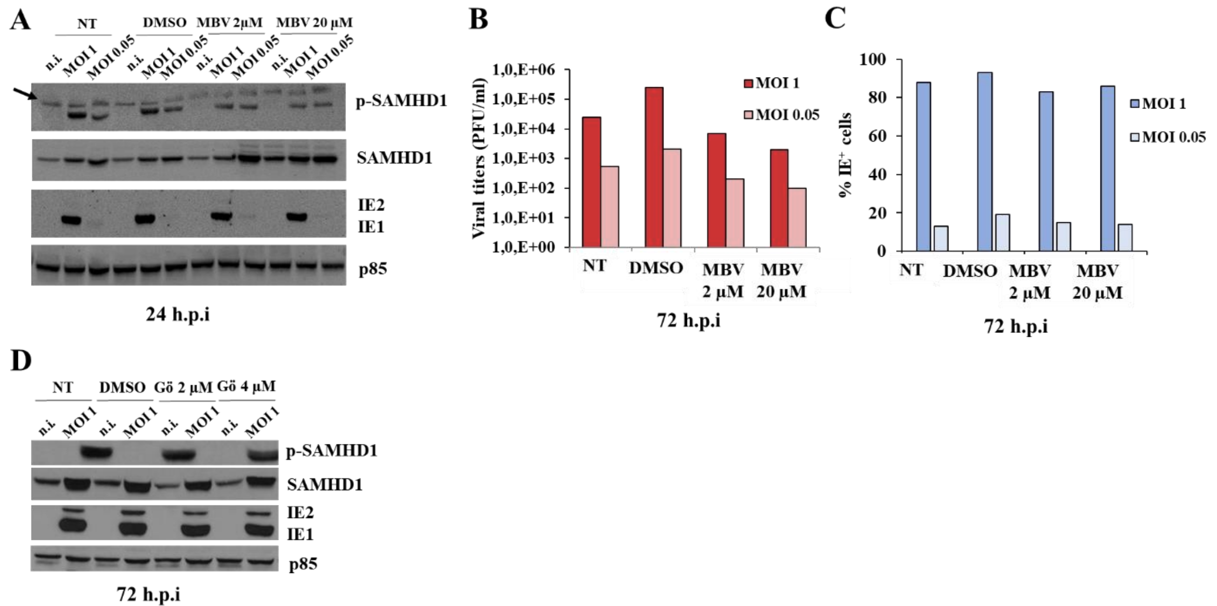

Figure 13. Effect of pUL97 viral kinase inhibitors on SAMHD1 phosphorylation and HCMV replication

HFFs were not infected (n.i.) or infected with AD169 at a MOI of 1 or 0.05, in the presence of different concentrations of the pUL97 chemical inhibitors Maribavir (MBV) and Gö6976 (Gö), or DMSO as control. After infection, the inhibitors were re-added in the cell culture until harvesting at the indicated times post-infection. A) Total or phosphorylated SAMHD1 levels were analyzed by immunoblot of HFFs treated with MBV at 2 µM or 20 µM and harvested at 24 h.p.i. IE1/IE2 was used as control of infection, and p85 as loading control. One representative experiment out of 2 performed at times post-infection ranging from 8 h.p.i to 6 d.p.i is shown. Arrow: unspecific band. B) Viral titers in the supernatant of infected HFFs treated with 2 µM or 20 µM of MBV were measured by standard plaque assays at 72 h.p.i. C) IE$^+$ cells percentage was analyzed by FACS after intracellular staining at 72 h.p.i. with a specific anti-IE1/IE2 antibody. Results are expressed as means, and one representative experiment out of 2 is shown. D) Total or phosphorylated SAMHD1 levels were analyzed as in panel A in cells treated with Gö6976 (Gö). One representative experiment out of 2 performed at 72 h.p.i is shown. NT, not treated; DMSO, cells treated with the vehicle DMSO.

5. The cellular Cdk1 inhibitor CGP74514A reduces HCMV replication and abrogates SAMHD1 T592 phosphorylation

pUL97 possesses similar functions of cellular Cdks and, together, they have redundant roles in phosphorylating cellular targets related to cell cycle regulation (Prichard, 2009). Notably, Cdk1 has been described to interact with SAMHD1 and to phosphorylate it at the T592 site, destabilizing the active tetramer and leading to a cellular environment permissive for HIV-1 infection (Cribier et al., 2013; Gelais et al., 2014). We then hypothesized that after HCMV infection, SAMHD1 could be phosphorylated by Cdk1 and this could facilitate HCMV replication.

To test this hypothesis, we infected HFFs with AD169 at a MOI of 1 or MOI 0.05, in the presence of the Cdk1 inhibitor CGP74514A, or with DMSO as control. We then evaluated SAMHD1 T592 phosphorylation levels by immunoblot, and viral titers by plaque assay (**Figure 14**). CGP treatment showed a dramatic reduction of SAMHD1 phosphorylation, compared to untreated or DMSO-treated control cells, both at 24 h.p.i (**Figure 14A**) and 72 h.p.i (**Figure 14B**) and at either of the two concentrations used. Interestingly, while the percentage of IE^{+} cells infected at an MOI of 1 showed only a marginal decrease (data not shown), plaque assays showed a dramatic reduction in the number of plaques in CGP-treated compared to DMSO-treated cells (**Figure 14C**), resulting in a ~100-fold decrease of HCMV plaque forming units (PFU) (**Figure 14D)**.

Together, these results suggest that Cdk1 can phosphorylate SAMHD1 in the context of HCMV infection, and that the CGP-induced inhibition of HCMV replication occurs after IE expression, during later stages of infection, and it could be in part SAMHD1-mediated.

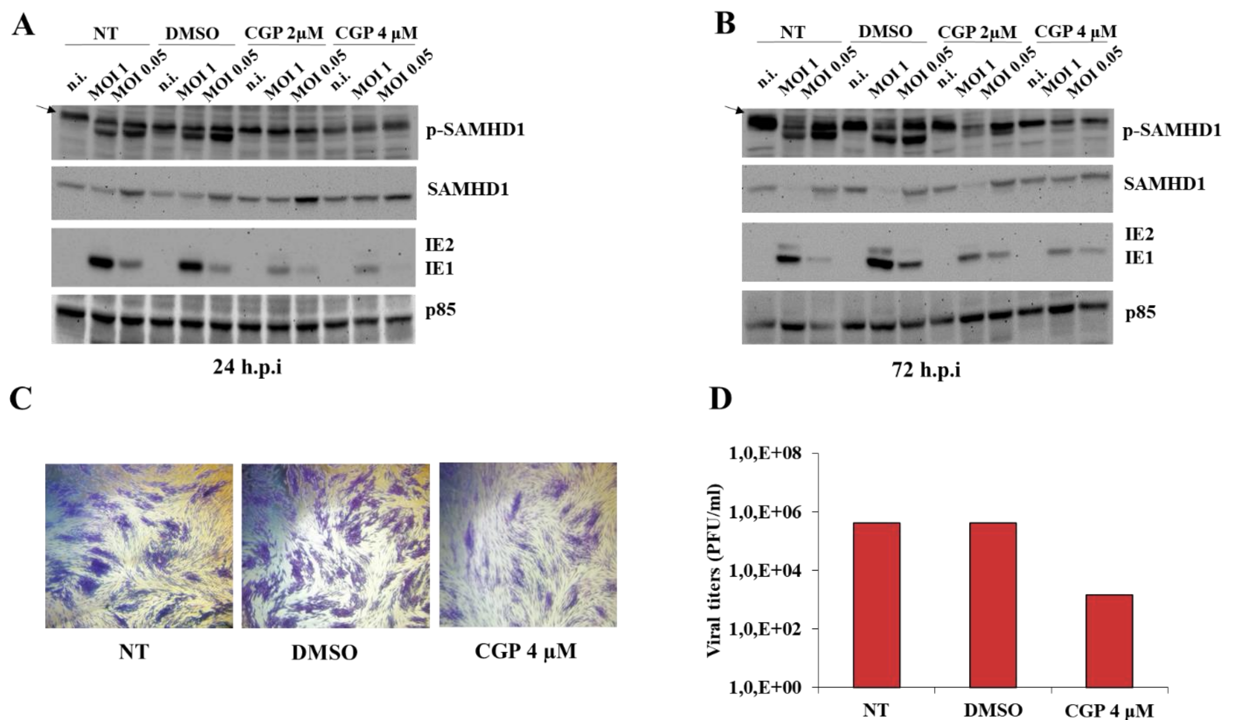

Figure 14. Effect of the cellular Cdk1 inhibitor CGP74514A on SAMHD1 phosphorylation and HCMV replication

HFFs were not infected (n.i.) or infected with AD169 at a MOI of 1 or MOI 0.05, in the presence of the Cdk1 inhibitor CGP74514A (CGP) at 2 μM or 4 μM, or DMSO used as control. After infection, CGP was re-added in the cell culture until harvesting. A-B) Total or phosphorylated SAMHD1 levels were analyzed by immunoblot of HFFs treated with CGP and harvested at 24 h.p.i (A) or 72 h.p.i (B). IE1/IE2 was used as control of infection and p85 as loading control. Arrow: unspecific band. C-D) Viral titers in the supernatants of cells untreated (NT), or treated with DMSO or with CGP at 4 μM were measured by standard plaque assays at 72 h.p.i. For both panels, one representative experiment out of 7 is shown. NT, not treated; DMSO, cells treated with the vehicle DMSO.

6. HFFs overexpressing wild type or T592 SAMHD1 mutants are equally permissive to HCMV replication

Since the T592 residue is an important site of regulation, the use of phospho-mimetic and/or phospho-defective mutants is a useful approach to unveil molecular features and functions of this site (Arnold et al., 2015; Welbourn et al., 2013).

We thus investigated if overexpression of wild type SAMHD1 or of T592 mutants could affect HCMV replication. HFFs were infected with retroviral vectors containing pOz plasmids encoding SAMHD1 wild type (wt), phosphodefective/constitutively active (T592A), or phosphomimetic/constitutively inactive (T592E) mutants. Three days later, cells were infected with AD169 at a MOI of 1 or 0.05 and SAMHD1 expression and viral titers were evaluated at 72 h.p.i and 6 d.p.i, respectively (**Figure 15**).

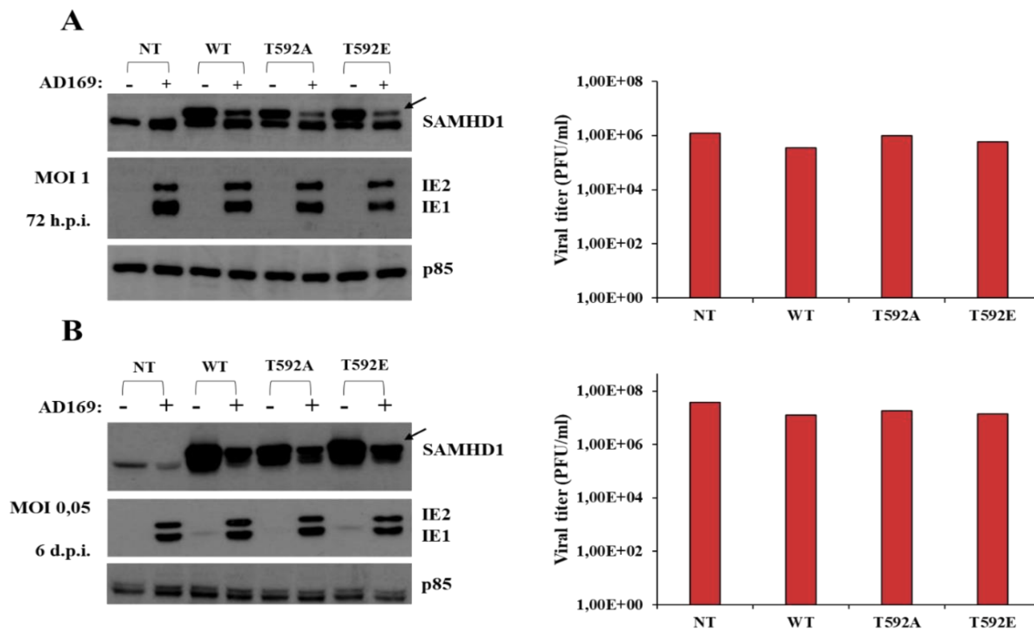

Figure 15. HCMV replication is not influenced by the overexpression of wild-type or T592 SAMHD1 mutants

A) HFFs transiently overexpressing wild-type or T592 SAMHD1 mutants were not infected (-) or infected (+) with AD169 at a MOI of 1. SAMHD1 overexpression was analyzed by immunoblot at 72 h.p.i. IE1/IE2 was used as control of infection, and p85 as loading control. One representative experiment out of 3 is shown (left panel). Viral titers in the cell culture supernatants of the same experiment were measured by standard plaque assays (right panel). B) SAMHD1 overexpression in uninfected or infected cells at MOI of 0.05 was analyzed at 6 d.p.i as in panel A. One representative experiment out of 7 is shown (left panel). Viral titers in the cell culture supernatants of the same experiment were measured by standard plaque assays (right panel). NT, not treated; WT, wild-type; T592A, phospho-defective mutant; T592E, phospho-mimetic mutant. The arrow indicates the overexpressed exogenous SAMHD1, migrating at a higher molecular weight than endogenous SAMHD1.

Overexpression of wild type or of T592 mutants did not change viral titer production at either time post-infection (**Figure 15, right panels**), suggesting that SAMHD1 phosphorylation is not involved in the modulation of a restriction activity against HCMV. Moreover, immunoblot analysis showed an equal expression of IE1/IE2 proteins with all the constructs tested (**Figure 15, left panels**). Of note, infected cells showed lower amounts of exogenous SAMHD1 constructs, that are HA- and FLAG-tagged and correspond to a higher molecular weight band, compared to endogenous SAMHD1 (**Figure 15,** black arrow in left panels). Since all retroviral constructs were almost equally expressed in uninfected cells, we conclude that the decrease of exogenous SAMHD1 expression was somehow triggered by HCMV infection, though for reasons that at present

are still unknown. These results suggest that SAMHD1 overexpression cannot limit IE protein expression and viral replication, independently from its phosphorylation status.

7. Phospho-T592-SAMHD1 preferentially localizes in the cytoplasm after HCMV infection

SAMHD1 is a nuclear protein, and in this site it acts as a dNTP pool regulator and HIV-1 restriction factor (Goldstone et al., 2011; Hrecka et al., 2011; Laguette et al., 2011). Recent observations reported SAMHD1 as a protein whose nucleocytoplasmic shuttling is important to suppress long interspersed element-1 (LINE-1) reverse transcription and transposition, by a distinct but still unknown mechanism than dNTP pool regulation (Du et al., 2019). Moreover, HCMV infection causes, among others, phosphorylation and cytoplasmic re-localization of the restriction factor IFI16, in a mechanism of evasion from intrinsic immune responses (Dell'Oste et al., 2014). We thus hypothesized that HCMV could be able to subvert the nuclear/cytoplasmic shuttling dynamic of SAMHD1 and re-localize it in the cytoplasm in order to evade its functions. To test this hypothesis, we firstly infected HFFs with AD169 and investigated total and p-T592-SAMHD1 localization at 72 h.p.i. by confocal microscopy analysis (**Figure 16**).

In uninfected cells, the analysis confirmed the constitutive expression of total SAMHD1 (**a**) and the absence of T592 phosphorylation (**g**). Upon infection, total SAMHD1 expression increased (**d**) and there was an induction of p-T592 levels (**j**). These observations are in line with immunoblot analysis (**Figure 9 and Figure 12**). Immunostaining of IE proteins showed their nuclear localization (**e**) (**k**), as previously reported (Lafemina et al., 1989). With our surprise, upon HCMV infection pT592-SAMHD1 was preferentially localized throughout the cytoplasm (**Figure 16A**) (**j**) (**l**).

To confirm the observation of pT592-SAMHD1 localization in the cytoplasm, we performed a nucleus/cytoplasm fractionation experiment of uninfected and infected cells at 72 h.p.i. Immunoblot analysis showed, as expected, a nuclear localization of total SAMHD1 in uninfected cells, but also a distinct presence in the cytoplasm, despite densitometric analysis did not reveal a statistically significant difference between the two compartments (**Figure 16B-C**). p-T592-SAMHD1 was undetectable in uninfected cells, in line with our previous results (**Figure 12**) and, upon infection, it was detectable in both

fractions, though it was prevalently localized in the cytoplasm (**Figure 16B**), with a statistically significant difference compared to nuclear compartment (**Figure 16C**).

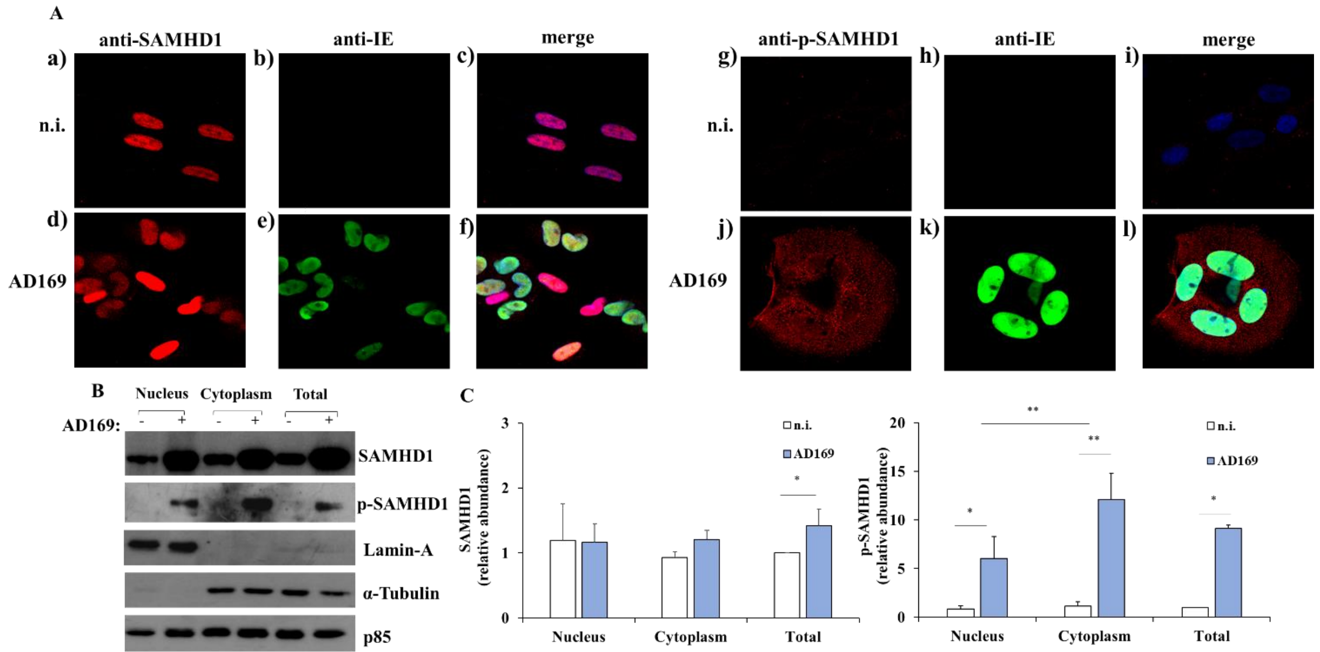

Figure 16. SAMHD1 intracellular localization upon HCMV infection
HFFs were not infected (n.i.) or infected with AD169 at a MOI of 1 for 3 days. A) Confocal microscopy stainings were performed using specific primary antibodies directed against IE1/IE2, total SAMHD1 or phospho-SAMHD1. Nuclei were stained with DAPI. One representative experiment out of 7 is shown. Red: total and phospho-SAMHD1; green: IE1/IE2; blue: DAPI. B) Total or pT592-SAMHD1 was analyzed in nuclear, cytoplasmatic and total cell lysates by immunoblot of HFFs not infected (-) or infected with AD169 (+). Lamin-A and tubulin were used as control of the purity of nuclear and cytoplasmic fractions, respectively. One representative experiment out of 5 performed at 72 h.p.i is shown. C) The relative abundance of total and phospho-T592 SAMHD1, normalized to p85 expression, was determined by densitometric analysis and it is relative to that of total cell extracts in n.i. cells, arbitrarily set as 1. Data are expressed as mean and error bars represent the standard error calculated from 4 independent experiments. *p* values were calculated by a Student *t* test. *, $p < 0.05$; **, $p < 0.01$.

Collectively, these results confirm with *in vitro* imaging experiments the increase of SAMHD1 protein expression and phosphorylation after infection and indicate for the first time that a large fraction of phospho-T592-SAMHD1 localizes in the cytoplasm of HCMV-infected cells.

8. SAMHD1 association with HCMV infectious particles, non-infectious dense bodies and viral proteins

The unexpected observation of SAMHD1 localization outside the nucleus upon HCMV infection led us to hypothesize that the exit from the nucleus could be due to an interaction with viral structures. To further investigate this aspect, we performed an ultrastructural analysis on uninfected and infected cells, by using cryo-immunoelectron microscopy (Cryo-IEM) (**Figure 17**). This technique showed that SAMHD1 was localized in the nucleus, and partially also in cytoplasm of uninfected cells (**a**). Upon HCMV infection, the gold labels associated to SAMHD1 maintained the same distribution and did not associate with viral particles (**b,** see the arrow). On the other side, cryo-IEM of phospho-T592-SAMHD1 showed minimal labelling in the nucleus and in the cytoplasm of uninfected cells, as expected (**c**). Indeed, upon infection, phospho-T592-SAMHD1 was associated with infectious viral particles and non-infectious dense bodies (**d,** see the arrow). These results confirmed the cytoplasmic localization of pT592-SAMHD1 upon HCMV infection and reported for the first time the association with HCMV viral particles and non-infectious dense bodies.

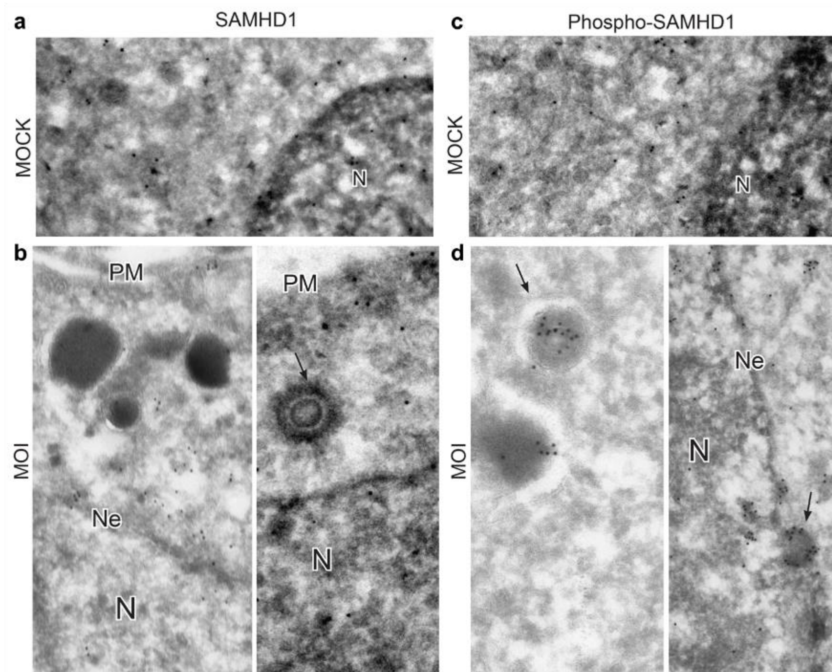

Figure 17. P-SAMHD1 association with viral particles
HFFs were not infected (n.i.) or infected with HCMV laboratory AD169 at a MOI of 1. At 72 h.p.i., cryo-IEM analysis was performed using specific primary antibodies against SAMHD1 (**a, b**) and p-SAMHD1 (**c, d**). One representative experiment out of 3 is shown. **N**, nucleus; **PM**, plasma membrane; **Ne**, nuclear membrane. Arrows: infectious viral particle.

Given the observation of SAMHD1 interaction with HCMV structures, we investigated if and which HCMV protein(s) could be able to interact with SAMHD1 and thus potentially modulate its localization after infection. To answer this question, we infected HFFs with AD169 and, at 72 h.p.i, we immunoprecipitated SAMHD1 and analyzed its interacting viral partners by mass spectrometry. In a preliminary experiment, we identified 5 viral proteins interacting with SAMHD1 (**Table 4**).

Table 4. SAMHD1 interacting HCMV proteins identified in HFFs by mass spectrometry

Protein name	Size (kDa)	Gene Name	Uniprot ID
Cytoplasmic envelopment protein 2	38	UL94	P16800
Small capsomere-interacting protein	8	SCP	Q7M6N6
Major DNA-binding protein	133	DBP	P17147
Transcriptional regulator UL34	45	UL34	P16812
Protein UL84	65	UL84	P16727

Three of them are associated with the assembly of viral particles (UL94, SCP, DBP), one with transcription of viral genes (UL34) and one with viral genome replication (UL84). These preliminary results indicate that SAMHD1 can interact with several HCMV-encoded proteins, probably mediating its association with viral particles and cytoplasmic re-localization.

9. Discussion

SAMHD1 has been established as a central regulator of dNTP metabolism. To date, it is the only known cellular enzyme hydrolysing dNTPs into dNs and inorganic triphosphates (Goldstone et al., 2011), and thus, the only one that negatively balances the activity of cellular ribonucleotide reductase (RNR), reducing the pool of available dNTPs for DNA synthesis. Since dNTPs are essential for cellular as well as viral DNA synthesis, SAMHD1 emerged in 2011 as a key restriction factor for retroviruses, particularly HIV-1 (Hrecka et al., 2011; Laguette et al., 2011). Since then, despite its role in retroviral restriction remains the most explored, SAMHD1 has been reported to restrict an increasing number of DNA viruses (e.g. HBV, EBV, VACV and HSV-1) (Chen et al., 2014; Hollenbaugh et al., 2013; Kim et al., 2013; Zhang et al., 2019). However, since there were no studies published on HCMV, our purpose was to understand and deepening the possible restrictive role of SAMHD1 during HCMV infection.

SAMHD1 is widely expressed in almost all human tissues and cells, comprising fibroblasts (Schmidt et al., 2015) and in fact our data demonstrate a constitutive expression in primary human foreskin fibroblasts (HFFs), as well as in epithelial (ARPE-19) and endothelial cells (HMVEC) (data not shown). Our results also indicate that SAMHD1 mRNA and protein expression increases in fibroblasts starting from early stages of HCMV infection (**Figure 8 and Figure 9**), as well as in epithelial and endothelial cells, infected with either high-passaged or low-passaged HCMV strains (**Figure 9** and data not shown). The increase of SAMHD1 expression is therefore strain- and cell type-independent.

Our results also indicate that SAMHD1 has a modest effect in limiting HCMV replication. In fact, silencing of SAMHD1 mRNA or Vpx-mediated knocking-down of the protein, did not cause a statistically significant rescue in HCMV virion production, or an increase in the percentage of IE^+ cells (**Figure 10 and 11**). These results suggest that restriction factors and/or intracellular sensors of viral infections could be redundant and could cooperate to limit HCMV replication. Another possibility is that SAMHD1 can be modulated during very early stages of the infection and quickly inactivated, thus masking the effect of its knocking-down. At this regard, our results demonstrate that HCMV infection triggers a dramatic induction of T592 phosphorylation, already at early times post-infection and up to 96 h.p.i, and independently from the viral strain used (**Figure 12**). Indeed, SAMHD1 has

been reported to be regulated by different post-translational modifications, such as oxidation, acetylation or phosphorylation (Cribier et al., 2013; Lee et al., 2017; Mauney et al., 2017). Phosphorylation of T592 residue has been widely investigated as a mean to negatively regulate the retroviral restriction activity of the protein (Arnold et al., 2015; Bhattacharya et al., 2016; Cribier et al., 2013; White et al., 2013a). Despite T592 phosphorylation does not appear to influence SAMHD1 dNTPase activity (White et al., 2013a), it has been proposed to destabilize the tetrameric active form and impair its antiviral function (Arnold et al., 2015; Bhattacharya et al., 2016).

We then investigated the kinase involved in T592 phosphorylation, hypothesizing that the HCMV-encoded serine-threonine kinase pUL97 was the greatest candidate. In fact, pUL97 has been reported to be able to phosphorylate itself as well as viral and host proteins, comprising the antiviral restriction factor IFI16 (Dell'Oste et al., 2014; Prichard, 2009). Moreover, it is expressed already at 8-16 h.p.i, and is a nuclear protein, as SAMHD1 (Brandariz-Nuñez et al., 2012; Gill et al., 2012; Michel et al., 1996). Nevertheless, according to our results, viral pUL97 does not seem to phosphorylate SAMHD1. Treatments with the pUL97 inhibitors Maribavir and Gö6976 did not affect T592 phosphorylation, at any experimental condition tested (**Figure 13** and data not shown), despite the use of Maribavir led to a reduction of HCMV replication as previously reported (Biron et al., 2002).

On the other hand, treatment with CGP74514A, a chemical inhibitor of Cdk1, completely abrogated T592 phosphorylation, and at the same time reduced the efficiency of viral replication (**Figure 14**). CyclinA2/Cdk1 has been reported to interact with and phosphorylate T592 in *in vitro* kinase assays and in U937, HEK293T and THP-1 monocytic cells (Cribier et al., 2013; Gelais et al., 2014). These results let us to speculate that the reported retained Cdk1 activity during HCMV replication in infected cells (Sanchez et al., 2003) can result also in SAMHD1 inactivation mediated by T592 phosphorylation.

In order to further address the possible role of T592 phosphorylation in HCMV restriction, we overexpressed phosphodefective constitutively active (T592A), or phosphomimetic constitutively inactive (T592E) SAMHD1 mutants. Compared to the wild-type construct, ectopic overexpression of T592A or T592E mutants did not modulate production of infectious virions (**Figure 15**) thus indicating that SAMHD1 phosphorylation is not

involved in the modulation of a restriction activity against HCMV. Surprisingly, infection was always associated with a noticeable decrease of ectopic SAMHD1 (either wt or mutated), suggesting the possible involvement of an intracellular or a viral-encoded sensor of SAMHD1 overexpression beyond physiological levels, and the establishment of an unknown mechanism of downregulation. Thus, the eventual role of SAMHD1 phosphorylation during HCMV infection remains an interesting area of scrutiny that will need further experiments to be fully elucidated.

As mentioned above, SAMHD1 knock-down and, on the other hand, overexpression of wild-type and T592 mutants showed negligible influence on HCMV viral production, suggesting that SAMHD1 activity could be overcome by HCMV during lytic infection, independently from its phosphorylation status. We then investigated a possible mechanism of immune-evasion carried out by the virus. HCMV ability to establish persistent and life-long infections and the long co-evolution with its host, selected many ways to modulate and avoid immune responses, comprising relocation of nuclear restriction factors to the cytoplasm and seizure into viral tegument (Biolatti et al., 2018). In line with these observations, our results showed that HCMV-infected cells contain a considerable amount of pT592-SAMHD1 outside the nucleus in normal, uninfected cells (**Figure 16**). Cytoplasmic re-localization of SAMHD1 protein is unusual, and only two papers reported the same result, by immunoblot analysis of nuclear/cytoplasmic lysates (Baldauf et al., 2012; Du et al., 2019). Therefore, our results are the very first observation of pT592-SAMHD1 localization in the cytoplasm of infected cells, suggesting the possibility of a mechanism of viral immune evasion by delocalization.

This promising and unexpected result led us to deepen the phenomenon, analyzing SAMHD1 distribution in uninfected and infected cells by visualization of cellular cryo-ultramicrotomy sections with transmission electron microscope. The results confirmed our observation of pT592-SAMHD1 cytoplasmic localization after HCMV infection and revealed for the first time an association of the protein with HCMV viral particles and dense bodies (**Figure 17**). Dense bodies are non-lysosomal vesicles that buds from the viral assembly complex (VAC) and the Golgi apparatus (Craighead et al., 1972). They are capsidless and, similarly to infectious viral particles, they are associated with many different viral glycoproteins (Mocarski et al., 2013). Therefore, our hypothesis is that, upon HCMV infection, pT592-SAMHD1 shifts from a tetrameric active to a monomeric inactive

form, exposing the SAM domain, facilitating its interactions with viral proteins, infectious viral particles and/or non-infectious dense bodies, and promoting its exit from the nucleus. To further address this hypothesis, we performed mass spectrometry analysis of immunoprecipitated lysates from HCMV-infected cells, revealing that SAMHD1 could interact with UL94, SCP, DBP, UL34 and UL84 viral proteins (**Table 4**). Though very preliminary, these results suggest that these viral proteins could be involved in the association of SAMHD1 with viral particles. However, more experiments and further studies are needed to confirm and validate these results and to draw conclusions to their actual role in SAMHD1 egress and association with viral structures.

While this thesis was in preparation, a study on the same topic was published by Kim et al. (Kim et al., 2019). Kim et al. demonstrated that SAMHD1 exerted an antiviral activity by negatively regulating NF-kB activation and *IE1* gene expression, at early steps of viral replication. Moreover, Kim reported that pUL97 can interact with and phosphorylate T592 residue in *in vitro* kinase assay (Kim et al., 2019). Despite some similarity (e.g. SAMHD1 mRNA and protein level increase upon HCMV infection; induction of T592 phosphorylation; absence of a significant role of T592 phosphorylation during HCMV infection), our results extend their findings, particularly related to the SAMHD1 involvement in full replication of HCMV virions. We did not focus on the NF-kB pathway, but we did not observe a negative effect of SAMHD1 on IE1/IE2 protein expression. Furthermore, we demonstrated that SAMHD1 silencing or knock-down was associated with a very slight but reproducible viral titer increase (~2-fold). We thus speculate that some unknown mechanisms of evasion can settle at the post-transcriptional level of IE1/IE2 expression, relieving suppression of *IE1* gene expression and facilitating HCMV replication. Moreover, unlike Kim et al., we reported that pUL97 is not involved in T592 phosphorylation (**Figure 13**), maybe because cellular Cdks could phosphorylate SAMHD1 (Cribier et al., 2013; Pauls et al., 2014), and thus mask the impairment of pUL97 activity provided by the use of chemical inhibitors.

In short, these are like drops in the ocean. What is the biological meaning of SAMHD1 increase of expression upon HCMV infection? Are there mechanisms established by HCMV to exploit the activity of SAMHD1? Are there post-translational modifications of the protein manipulated by the virus to favor its replication? The answers to these questions will be probably potential opportunities to deepen SAMHD1 role in innate

immunity and enrich the plethora of mechanisms acted by HCMV to establish latent and life-long infections. The hope for the future is that the research work in this field will contribute to develop good therapeutic strategies against HCMV and/or develop a cure or a prophylactic vaccine, that are still missing.

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