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Characterization of host citrullination during Human Cytomegalovirus infection and its potential for novel antiviral drugs

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UNIVERSITY OF TURIN

PhD School in Life and Health Sciences
Molecular Medicine

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INTRODUCTION

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1 Human cytomegalovirus

1.1 General features

The *Herpesviridae* (HV) family consists of large double-stranded DNA (dsDNA) viruses that infect and cause diseases in species all across the animal kingdom, in mammals, birds, reptiles, amphibians, fish, and invertebrates[1]. Based on phylogenetic analysis, it was estimated that mammalian HVs arose 400 million years ago from an ancient common ancestor[2].

Although HVs express non-coding RNAs and miRNAs, protein-coding regions occupy the great majority of their large genomes[1]. HVs coding genes are generally divided into "core" genes, which are shared by all HVs due to inheritance from a common ancestor, and "non-core" genes[1]. Most of the core genes encode proteins essential for viral DNA replication, viral DNA packaging and capsid structure and assembly. Herpesviruses have evolved through nucleotide substitution, genetic rearrangements, recombination, gene duplication and capture. Non-core genes are specific to HVs genera or even species and, in contrast to core genes, are often dispensable for viral growth in cell culture[1]. Nonetheless, many non-core genes have important roles for growth *in vivo*, indicating that most genes contribute to the success of HVs in nature, whether or not an associated phenotype can be detected in the laboratory setting[1].

Nine members of this group of viruses infect humans as their primary host[3] (Table 1). Herpesviruses are distinguished into three subfamilies based on shared biological characteristics and genome sequence phylogeny: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*[4]. The *Alphaherpesvirinae* have a neuronal tropism and establish latency in neuronal ganglia. They show extensive host species range *in vitro.* Herpes simplex virus types 1 and 2 and varicella-zoster virus (HSV-1, HSV-2 and VZV) are the most important representatives of this family. The *Betaherpesvirinae* are characterized by slow replication in cell culture and limited host range. They establish latency in peripheral blood monocytes[5,6]. Members include cytomegaloviruses (CMVs) such as HCMV (also known as human herpesvirus 5 (HHV-5)) and also human herpesviruses 6 and 7 (HHV-6 and HHV-7). The *Gammaherpesvirinae* are lymphotropic and they establish latency in lymphocytes. Members include Kaposi's sarcoma-associated herpesvirus (KSHV) (which is also known as human herpesvirus 8 (HHV-8)) and Epstein-Barr virus (EBV).

Herpesvirus genomes differ greatly in size[7]. Their GC-content (guanine-cytosine content) also varies considerably, ranging from 32-75 %[8].

Formal name	Common name	Subfamily	Site of latency
HHV-1	HERPES SIMPLEX-1 (HSV-1)	α	Neurons
HHV-2	HERPES SIMPLEX-2 (HSV-2)	α	Neurons
HHV-3	VARICELLA ZOSTER (VZV)	α	Neurons
HHV-4	EPSTEIN-BARR VIRUS (EBV)	γ	B Lymphocytes
HHV-5	CYTOMEGALOVIRUS (HCMV)	β	Myeloid lineage
HHV-6A		β	T lymphocytes
HHV-6B		β	T lymphocytes
HHV-7		β	T lymphocytes
HHV-8	KAPOSI's SARCOMA ASSOCIATED HERPES VIRUS (KSHV)	γ	B Lymphocytes

Table 1 Schema of the nine members of *Herpesviridae* family infecting humans as primary host.

These viruses are among the most successful human pathogens in terms of global distribution, persistence in the host, and transmissibility. HVs infection of immunocompetent human subjects generally results in a mild disease, although severe consequences may develop when infection occurs in immunocompromised subjects.

Our main working model in the lab is the human cytomegalovirus (HCMV). Human cytomegalovirus (HCMV) is a member of the *Betaherpesvirinae* subfamily (Table 1), whose virion structure, kinetics of viral gene expression, and persistence for the lifetime of their host are typical of other herpesviruses. The name is derived from the fact that it causes enlargement of the infected cell (cytomegaly) and induces characteristic inclusion bodies. Compared to other human herpesviruses, HCMV is the largest, with a genome of ~235 kb encoding~170 genes. The virion consists of a double stranded linear DNA core in an icosahedral nucleocapsid, enveloped by a proteinaceous matrix (tegument). These components are enclosed in a lipid bilayer envelope that contains a number of viral glycoproteins (Figure 1)[9].

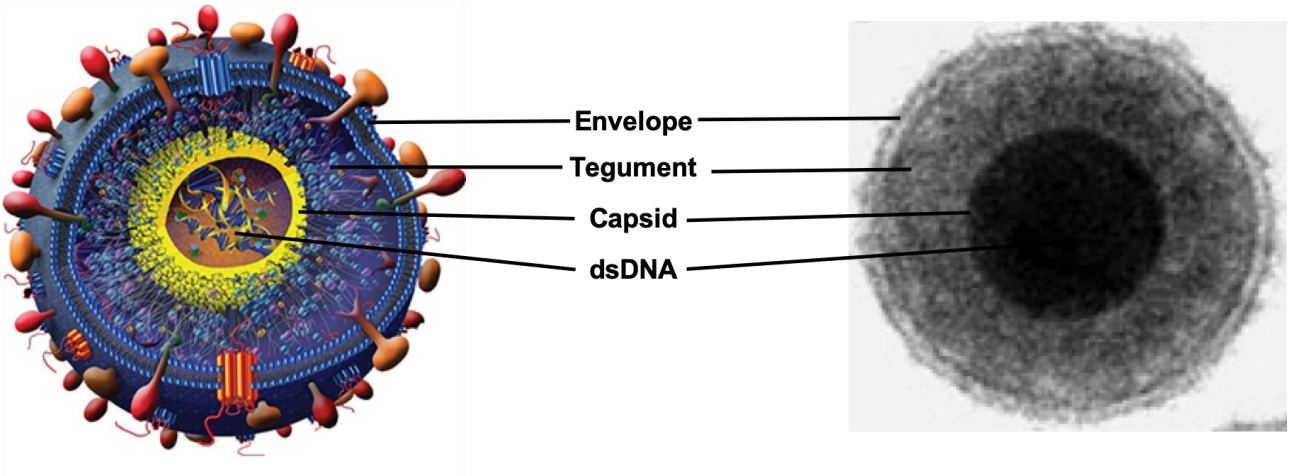

Figure 1 Virtual three-dimensional model of HCMV showing various components of the virus (left panel)[10] and electron microscopy of HCMV mature viron (right panel)[11].

The expression of HCMV genome is controlled by a cascade of transcriptional events that leads to the synthesis of three categories of viral proteins designated as immediate-early (IE), early (E), and late (L). Mature virions range in diameter from 200 to 300 nanometers. However, its strict species-specificity, salivary gland tropism, and slow growth in cell cultures, classify it as the prototype betaherpesvirus. It is a widespread pathogen that infects a majority of the world's population by early adulthood[3]. In fact, by the age of 40, between 50 and 85% of adults are infected by HCMV, a percentage that varies depending on the socio-economical background of each country[12]. The virus establishes a life-long infection with some cells being latently infected, a state where the virus has the ability to lie dormant within a cell, while others are persistently infected, where the infection cannot be cleared from an organism and there is intermittent shedding of infectious virions[13]. Immunocompetent individuals, who can develop a strong immune response, typically display no symptoms of infection[14]. However, in individuals whose immune systems are immature or weakened, such as organ-transplant recipients or acquired immunodeficiency syndrome (AIDS) patients, HCMV is a significant pathogen causing severe morbidity and mortality [15]. Symptoms in these individuals typically consist of spiking fever, leucopenia (decrease in white blood cells), malaise, hepatitis, pneumonia, gastrointestinal disease and/or retinitis (inflammation of the retina) [15]. HCMV is also responsible for approximately 8% of infectious mononucleosis cases and is the leading viral cause of birth defects often causing deafness and mental retardation, when the mother gets infected or re-infected during pregnancy[16,17].

HCMV has been implicated in inflammatory and proliferative diseases, including autoimmune diseases (AID), cardiovascular diseases and cancer[14]. Epidemiological and pathological studies have suggested a strong link between HCMV and atherosclerosis[18].

1.2 Viral structure

HCMV-infected cell cultures produce the infectious virions and other two types of morphological particles: non-infectious enveloped particles (NIEP) and dense bodies (DB). NIEP are defective viral particles composed of enveloped immature capsids (type B) that lack DNA, but contain the viral scaffolding/assembly protein (AP) normally absent in fully mature nucleocapsids (C-capsids). DB are enveloped particles that lack an assembled nucleocapsid and viral DNA, but contain several tegument proteins such as pp65 (pUL83 or lower matrix protein), that is the most abundant. The relative amounts of the three viral forms depends on the number of passages in cell culture and the viral strain[3].

HCMV-infected cells produce several types of nucleocapsid. Capsids are classified in type A, B and C. Gibson described type A capsids as dead-end products of abortive DNA packaging without viral DNA inside. Type B capsids lack also the DNA genome, but contain scaffolding proteins. Finally, type C nucleocapsids are mature DNA containing capsids[19].

1.1 HCMV capsid

Like other herpesviruses, HCMV capsid is organized in hexon capsomeres, penton capsomeres and triplex structures that connect the hexon and penton together. Furthermore, HCMV capsid consists of four structural proteins: the major capsid protein (MCP; pUL86), the minor capsid protein (mCP; pUL85), the minor capsid binding protein (mC-BP; pUL46) and the small capsid protein (SCP; pUL48A)[3]. Hexons and pentons are made by the MCP while the triplex proteins, that connect the capsomeres themselves are constituted by the mCP and the mC-BP. It has been shown that the SCP is fundamental for HCMV infection *in vivo* and also decorate the tips of the MCP hexon subunits[20,21]. In HCMV, tegument is not completely attached to the capsid[22]. The capsid is organized around the protein pUL80A that acts as a scaffold and binds three other proteins. All this complex is created by autoproteolytically cleaved pUL80, which contains protease activity in the amino-terminal portion. After capsid assembling the scaffold proteins are cleaved and the genomic DNA can then be packaged[23].

1.3 HCMV envelope

The HCMV lipid bilayer envelope derive from host intracellular membranes into which viral glycoproteins are inserted. Almost 50 glycoproteins are produced by HCMV, but the majority have not yet been studied, and it is not clear which are expressed on the host cell surface and/or in the

virion envelope[3]. Three major glycoprotein complexes are found in HCMV particles. Glycoprotein B (gpUL55) is a type 1 transmembrane protein that forms a homodimeric glycoprotein complex termed gcI. This complex interact with heparan sulphate proteoglycans to facilitate virus entry[8]. Results of sequence analysis show that at least four gB subtypes exist, and they display a sequence variation that is highest between codons 448 to 480[24]. Such sequence variation is probably linked to immune evasion, as approximately all individuals develop anti-gB neutralizing antibodies, reaching a neutralization efficiency of 50 % at most[25]. Glycoprotein complex gcII consists of the proteins gM (gpUL100) and gN (gpUL73), which form a gM/gN disulphide linked dimer and has heparin-binding activity, thus playing a role in virus entry and membrane fusion[26,27]. These genes are essential for HCMV replication in fibroblasts[27,28]. It has been demonstrated that the disulphide bond between gM and gN molecules was not necessary for complex formation or translocation of the gCII complex into the assembly compartments[29]. However, disruption of the C-terminal domain of gN led to a reduction of secondary envelopment of HCMV capsids, suggesting a critical role for gN in mediating this process[30]. Glycoprotein complex gcIII consists of gH (gpUL75), gL (gpUL115) and gO (gpUL74), and is needed for viral entry into host cells, since antibodies that mime the gH/gL complex and bind to the same cell surface receptor as gCIII prevent HCMV from penetrating the cell membrane[31]. When gB or gH binds the cell surface receptors, an intracellular signaling cascade is induced resulting in the expression of the cellular transcription factors Sp1 and NF-κB[32].

1.4 HCMV tegument

The tegument is a multifunctional structural complex component of the virion. Tegument proteins have several functions that include: virus egress, particle architecture, trans-activation of IE gene expression, and arrangement of the cellular environment for lytic replication. pp65 (pUL83) and pp150 (pUL32) are the most abundant tegument proteins comprising about the 20 % of the total viral particle protein mass[33,34]. Despite its abundance, pp65 is not essential for growth in tissue culture[35]. After infection, particle-delivered pp65 localizes into the nucleus. Expression of the UL83 (pp65) gene occurs at early-late times post infection, and *de novo* synthesized pp65 is localized in the nucleus throughout the replication cycle, but at late times post infection, is also accumulated in the cytoplasm[36]. It was reported that pp65 has serine/threonine protein kinase activity capable of both autophosphorylation and phosphorylation of IE1[37]. It was also shown that pp65 prevents cleavage of IE1, inhibiting the presentation of IE1 peptides via MHC class I[37]. The association of pp65 with a cellular protein, Polo-like kinase (PIK1) affects its intracellular signaling, sub-cellular location and substrate specificity[38]. The basic-phosphoprotein pp150 is an immunodominant protein, that lead to the development of antibodies found in 85 % to 90 % of HCMV seropositive individuals[3]. Its

modification with an O-linked N-acetylglucosamine associates with nuclear localization. pp65 and pp150 are localized in the nucleus at early times post infection, but at late times of infection they are found in the cytoplasm[36]. On contrary, few studies detected pp150 exclusively in the cytoplasm[39]. As pp150 binds the MCP, it is possible that pp150 is involved in tegument attachment[40].

Other tegument proteins include the transactivators pp71 (pUL82), pUL69, pTRS1/pIRS1, which activate the virus IE promoter/enhancer and other virus and cellular promoters. The tegument is also reported to contain multiple members of the US22 gene family: pUL23, pUL24, pUL43, pUS22; pUL36, pTRS1/pIRS1; pUS23 and pUS24[3,41–43]. pUL99 (pp28) is also a tegument protein and was reported to be essential for virus replication and envelopment of HCMV capsids[44]. Together, these data suggest a complex role for tegument proteins from initiating lytic infection, evading immune defenses and/or modifying the cellular environment, to maturation and egress of virions.

1.2 Viral genome

The HCMV genome is the largest of all herpesviruses and has a high G+C content.

It consists of two covalently-linked region, unique long (UL), unique short (US), each flanked by inverted repeats (Fig. 2) [Long Terminal Repeat (TRL) and Long Internal Repeat (IRL), and Short Terminal Repeat (TRS) and Short Internal Repeat (IRS) respectively]. Architecture of herpesvirus genome can be divided in six classes from A to F (Roizman, Family Herpesviridae). Since each long and short region can be oriented in either direction, four genome isomers are produced in viral progeny (Class E structure). In contrast, the genomes of animal CMV, as well as those of other betaherpesviruses, are linear without repeat regions (Class F genomes). Inversion of UL and US regions is mediated by direct repeat sequences (**a, b, c**) at the genome termini and by inverted repeat elements at the UL-US junction (**a′, b′, c′**). The repeated **a** sequence promotes genome isomerization, since it contains the cis-acting pac (packaging) elements needed for DNA cleavage and packaging of progeny during viral replication[3].

HCMV is the largest and most complex of the nine human herpesviruses. The DNA sequence of strain AD169 was the first complete HCMV genome to be published[45]. Analysis of the AD169 laboratory strain has revealed that its 235 Kbp genome encodes 225 ORFs of ~100 or more aminoacids[45,46]. These ORFs are designated sequentially according to their location within the unique and repeated regions (Fig. 2). Additional ORFs have been identified in the Towne and Toledo laboratory strains. In the latter, as well as in clinical isolates, the inverted **b′** repeat is deleted and replaced by an additional UL region of ~15 kbp, containing 19 additional ORFs that are absent in the AD169 genome[47]. The unique ORF UL1−154 and US1−36 blocks are separated by duplicated IRL1−14 and

J1I genes and the partially repeated IRS1 gene. The UL region is flanked at the 5′ end by the duplicated TRL1– 14 and J1L (identical to IRL1–14 and J1I), whereas the US gene block is flanked at its 3′ end by the TRS1 gene and by the third copy of a J1 gene (J1S). With technological advancement, the coding potential of HCMV is beginning to be understood better. Recent high-definition analyses of the HCMV transcriptome using RNA-Seq, RNA mapping, and ribosome profiling have revealed that gene expression in HCMV is even more complex than previously thought, in part owing to highly regulated non-canonical translation patterns, overlapping ORFs, and use of non-conventional initiation codons[48,49]. One study has identified over 600 additional protein coding ORFs, the majority of which are very short and situated upstream of longer ORFs[49]. In addition to protein coding genes, HCMV also produces polyadenylated non-coding RNAs, including four long non-coding RNAs – RNA2.7, RNA1.2, RNA4.9, and RNA5.0 – that are produced in abundance and do not overlap with protein coding regions. Additional non-coding RNAs are transcribed anti-sense of protein coding regions[48,50]. Non-polyadenylated HCMV RNAs include microRNAs, which are involved in numerous processes related to regulating host cell metabolism, immune evasion, and maintenance of latency[51–56].

Comparison of the AD169 aminoacid sequences with those of other herpesvirus genomes has revealed that the protein products of more than 40 ORFs share high similarity to proteins encoded by α- and γ-herpesviruses, and provided further evidence of a common origin of the three subfamilies. Of the herpesvirus conserved ORFs, ~25% appear to encode functions related to viral DNA metabolism and replication, whereas the remaining 75% are thought to be involved in the maturation and structural organization of virions[45]. The UL ORF-encoding functions involved in DNA replication and repair, nucleotide metabolism, or virion structure are grouped in seven conserved gene blocks (A–G) also found in other herpesviruses, such as HSV-1 and EBV virus, although in a different order than in the α- and γ-herpesviruses. The occurrence of these conserved blocks in a characteristic order specific to each subfamily suggests that the functions encoded are probably conserved in all herpesviruses. The US ORF and those located within the repeated regions of the HCMV genome are less well conserved than in the other herpesviruses[45].

Sequence homology searches and experimental biochemical and/or genetic studies have assigned functional roles to only some of the more than 200 HCMV ORFs[46]. However, analysis of the phenotypes of spontaneous deletion mutants of the AD169 strain, as well as those of virus-bearing deletions or inactivation at specific loci, has indicated that the products of more than 50 HCMV ORFs are dispensable for productive replication in fibroblast cultures. These findings, along with observations that the proteins responsible for functions common to all herpesviruses, such as basic DNA replication, virion organization, and maturation, do not account for all the ORFs, indicate that many ORFs still await functional characterization. It is likely, therefore, that much of the coding

capability has evolved to optimize infection by influencing dissemination, growth in target tissues and pathogenesis, and in counteracting host immune reactivity[3].

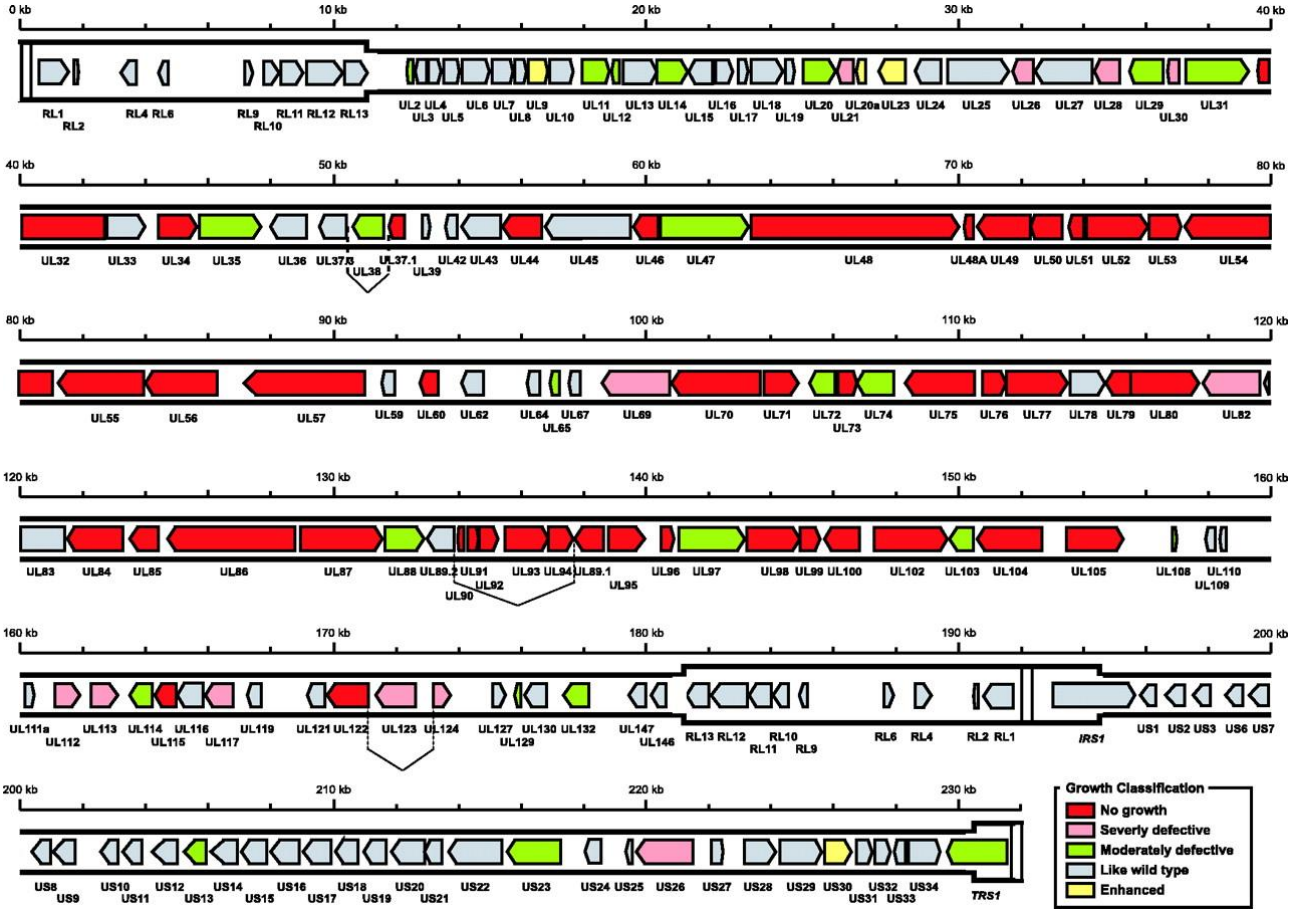

Figure 2

Genome organization and ORF dog HCMV (Towne strain) based on the genomewide shotgun sequencing of the viral sequence cloned in a BAC.
It is composed of a UL region and a US region, both flanked by inverted repeat regions (RL and RS). RL and RS are shown in a thicker format than UL and US. Each of the ORFs (RL1-RL13, UL2-UL147, IRS1, US1-US34, and TRS1) is color-coded according to the growth properties of their corresponding virus gene-deletion mutants in HFF. The vertical dashed lines represent the splicing junctions[57].

1.5 Replication cycle

1.5.1 Virus binding and entry

Virus attachment and penetration are rapid and efficient in both permissive and non-permissive cell types. However, since productive replication is observed in very restricted range of human cells, a post- penetration block to viral gene expression is thought to restrict replication in non-permissive cells[58]. The poorly characterized receptors for HCMV are widely distributed among host cells, and contributes to the broad viral tropism observed during natural infections. Viral entry is the result of a cascade of interactions between viral and cellular proteins that culminates in fusion of the virion

envelope with the cellular plasma membrane by a pH-independent mechanism. During the initial virus-cell interactions, as observed with other herpesviruses, HCMV attaches to the cell surface by low-affinity binding of gB to heparan sulfate proteoglycans[59]. The subsequent interaction of gB with its non-heparan receptor then turns the weak adhesion of the viral particle into a more stable binding or docking state. More recently, both the epidermal growth factor receptor (EGFR) and a specific subset of cellular integrins have been identified as HCMV entry and signaling receptors[60].

However, final fusion of the viral envelope with the cell membrane to allow viral penetration is thought to require a further priming event mediated by the heteroligomeric gH-gL-gO complex with as yet unidentified receptors[61]. Fusion of the virus and cell membranes is followed by entry into the host cytoplasm of the nucleocapsid and tegument proteins, and their rapid translocation into the nucleus, where pp65 is detected < 1-hour post infection. Interaction of HCMV glycoproteins with their receptors is enough to generate an intracellular signal transduction pathway, leading to the alteration of cellular gene expression. Most changes in the profiles of host gene activity resemble those induced by binding of interferons to their receptors[62,63]. The specific viral ligand triggering this response is gB, and its interaction with its as yet unidentified receptor is thought to be the main mechanism by which HCMV modifies host cell gene expression in the very early phases of infection[60,64,65]. In addition, engagement of gB and gH with their receptors is sufficient to induce activity of the cellular transcription factors nuclear factor-kB (NF-kB) and Sp1[32].

1.5.2 Regulation of viral gene expression

During productive infection, the HCMV genome is expressed in a temporally coordinated and regulated cascade of transcriptional events that lead to the synthesis of three categories of viral proteins described as IE or α, E or β, and L or γ. In a non-permissive cell the expression of E genes with subsequent DNA replication or attachment/penetration may be restricted. Transcription of viral genes occurs in the nucleus by RNA polymerase II and the associated basal transcription machinery, with the intervention of host-encoded transcription factors whose activity may be stimulated by viral trans activators[3,66].

1.5.3 Characteristics and functions of the immediate-early proteins

HCMV gene expression initiates from a few IE proteins within 1-hour post infection without *de novo* protein synthesis. The IE genes include the major IE (MIE) UL122/123 genes (IE1 and IE2) and auxiliary genes, such as UL36-UL38, UL115-UL119, IRS1/TRS1, and US3. The MIE proteins, alone or in synergism, are required for subsequent expression by acting as trans-activators and auto-simulators of

viral genes. In addition, these proteins have a deep impact on host cell physiology since they regulate the expression of a large number of host-cell genes[66]. MIE proteins are encoded by the ie1/ie2 genes (UL122/123), whose expression is regulated by a complex enhancer-modulator element that functions in a tissue- and cell-type specific manner, and exerts its strong transcriptional activity through interactions with several host transcription factors whose binding sites are closely distributed within regulatory element[67,68]. An ~500-bp segment upstream from the TATA box of the i1/ie2 genes contains several repeat elements with binding sites for NF-κB, AP-1, Sp1, and CREAB/ATF[69]. Since their cognate DNA-binding activities are rapidly activated by HCMV infection, their binding to the corresponding sites is thought to contribute to the very strong activity of the MIE enhancer. The CREB/ATF and AP-1 sites are also responsive to the tegument trans-activators ppUL82[70]. In addition, NF-1, serum response factor, Elk-1, CCAAT/enhancer binding protein, and YY1 sites have been identified within the enhancer segment. The cell type- and differentiation state-specific enhancer activity is related to the availability of appropriate transcription factors in a specific cell type, and is thought to depend on the modulator region. This element spans ~500-bp upstream from the core of the MIE enhancer. In transient transfection assays, the modulator region represses the transcriptional activity of the enhancer in undifferentiated cell lines, although these findings could not be reproduced with mutagenized viruses in which the modulator element was removed[67,71].

1.5.4 Characteristics and functions of the early proteins

The expression of functional IE proteins is necessary to induce E or β genes[66]. They are divided into two subclasses: β1 (E) and β2 (E-L) according to their time of expression. B1 genes are transcribed within 4-8 hours post infection (hpi), β2 transcription 8-24 hpi. E genes encode mostly non-structural proteins, including viral DNA replication factors, repair enzymes, and proteins involved in immune evasion[3].

The expression profiles of microarrays of viral DNA recently have provided a temporal map of IE, E, and L genes in the entire viral genome[72]. Hybridization of such microarrays to cDNAs prepared from HCMV-infected cells treated with ganciclovir (GCV) to block viral DNA replication, which does not affect E genes expression, has revealed that 36% of the more than 150 ORFs scored positive for expression were unaffected by GCV and, therefore, classified as E. These E genes are not clustered close to each within HCMV genome. Unlike the genes in the UL region, most US genes show E class expression properties. However, several E genes, such as UL4, UL44, UL54, and UL 112/113, are also transcribed late in infection through several mechanisms, including activation of a promoter

different and independent from that transcriptionally active in the E times, initiation of transcription from a new start site, and alteration of the splicing pattern as infection proceeds[3,66].

It is believed that transcription of E genes is stimulated by IE2-86 alone or in cooperation with IE1-72 through transactivation of the corresponding promoters in a TATA box-dependent manner that requires both the host basal transcription initiation complex and sequence-specific transcription factors, such as CREB/ATF and Sp1[66]. Both E and L transcripts may have a polycistronic structure due to the relatively few polyadenylation signals in the genome that generate families of 3' co-terminal transcripts. In addition, studies on the expression of several E genes revealed that they are regulated by both transcriptional and post-transcription mechanisms[3].

Few E genes are involved in viral DNA replication: for example the UL112/113 family of DNA-binding proteins that contribute to organization of the so-called replication centers within the nucleus of infected cells, the viral DNA polymerase encoded by the UL54 ORF and the UL44 gene product that acts as a polymerase processivity factor[3]. Other E proteins, however, are involved in establishment of immune evasion in the productively infected cell, such as the glycoproteins encoded by US2 and US11, which bind the MHC Class I heavy chains and transport them in a retrograde fashion from the ER into the cytosol, where they are degraded by the proteasome[73,74]. The E-expressed US27 and US28 ORFs have homology to the chemokine (CC) receptors[45]. However, US28 alone is a receptor for the CC chemokines RANTES and monocyte chemoattractant peptide-1. It sequesters them from the extracellular environment by internalization and, thus, prevents elimination of HCMV-infected cells by chemokine-activated immune cells[75,76]. In addition, the E gene UL4 encodes an E structural glycoprotein (gp48). This is a non-essential component of the viral envelope, since a mutant virus disruption of the UL4 gene produces virus progeny without impaired replication kinetics[28].

1.5.5 Viral DNA replication

HCMV genome replication and packaging occur in the nucleus of the infected cells. Viral DNA synthesis begins later than 16 hpi. It requires the activities of essential and specific viral proteins and the active contribution of several cellular proteins[3].

Examination of CMV genome sequences has shown that, unlike other herpesviruses, CMV is not able to encode deoxyribonucleotide biosynthetic enzymes, such as thymidine kinase, dihydrofolate reductase, thymidylate synthase, and an active form of ribonucleotide reductase[45,77]. Thus, the virus must depend on the host cell metabolism to ensure a sufficient supply of dNTPs for its DNA replication, thereby it doesn't shut off host macromolecular synthesis, but stimulates cellular transcription and translation.

In regards, it has been reported that the early consequences of CMV infection are similar to those observed in serum-deprived cells exposed to growth factors[78]. Strategies exploited by CMV to stimulate the biosynthesis of DNA precursors include: Cdk2 nuclear translocation, cyclin E and B induction, pRb hyperphosphorylation, E2F-dependent transcription activation and activation of c-myc, c-jun, and cfos proto-oncogenes[79–83]. Moreover, there is a substantial increase in the expression of cellular enzymes involved in nucleotide metabolism, including thymidine kinase, ornithine decarboxylase and topoisomerase II, dihydrofolate reductase, folylpolyglutamate synthetase, thymidylate synthase, deoxycytidilate deaminase, and ribonucleotide reductase[84–88]. Despite the induction of an S phase-like state, CMV-infected cells fail to undergo cellular DNA replication and division as a result of blocks in cell cycle progression that prevent the host DNA replication machinery from competing with the virus for access to DNA precursors[78,89]. The ability of HCMV to stimulate the expression of cellular enzymes for DNA precursor synthesis is crucial for its productive replication in quiescent or terminally differentiated non-dividing cells.

Six herpesvirus-conserved ORFs in HCMV genome provide the core replication proteins for viral DNA replication. Among them, the single-stranded DNA-binding protein ppUL57 prevents the reannealing of DNA strands following unwinding by the helicase-primase complex. This, in turn, is composed by three subunits encoded by UL70, UL102 and UL105, the DNA polymerase encoded by UL54 together with the DNA polymerase processivity factor UL44 that prevents dissociation of UL54 from the template[3].

Replication also requires other viral proteins to maximize DNA replication, such as UL84, UL112/113, and UL114. UL84 encodes a 75-kDa phosphoprotein, which stably interacts with IE2-86, functions as an origin-specific initiator factor and stimulates the viral origin (oriLyt)-dependent DNA synthesis. The phosphoproteins encoded by the UL112/113 region regulate the establishment of the so-called “replication centers” corresponding to subnuclear sites of HCMV DNA synthesis. UL112/113 localize to small intranuclear globular sites representing the early precursors of the replication centers and recruit the core replication proteins and enzymes[90]. Finally, the protein encoded by UL114 expresses a functional uracil DNA glycosylase activity that appears to be required for efficient viral DNA replication in post-mitotic cells, since a mutant virus with a substitution mutation in UL114 showed a defect in transition to high-level, late-phase DNA replication[91]. IE proteins, such as the transactivators encoded by the ie1/ie2 and TRS1/IRS1 genes and those expressed by the UL36-38 region, are also required for transient complementation of oriLyt-dependent DNA synthesis, although their roles in this process are not known. HCMV DNA replication proceeds through initial circularization of the input genome within 4 hpi, followed by DNA synthesis via a bidirectional mechanism from a single origin (oriLyt) of replication that undergoes a switch to a late-

phase rolling circle[3]. The latter is responsible for most of the viral DNA produced during the late stages of infection in the form of large concatemeric replicating units lacking terminal fragments, that are subsequently cleaved into pieces that can be encapsulated. The oriLyt of HCMV is located within the UL region close to UL57[92]. It spans ~2000 bp, containing repeated nucleotide sequences, as well as transcription factor binding sites, and encodes a number of short transcripts. The integrity of this region is needed for efficient replication, when mutations targeting different repeated sequence elements occur, the initiation of viral DNA synthesis is dramatically reduced [3].

During the late stages of viral DNA replication, newly synthesized genomes mature through their inversion, cleavage, and packaging[93]. Inversion occurs in concatemeric units and leads to the generation of progeny genomes as a pool of four isomers that only differ in the orientation of their L and S components. Packaging of the genome into preformed B capsids then follows its cleavage at the essential cleavage/packaging signals pac1 and pac2. These sequences are highly conserved among the herpesvirus genomes and are contained within a 220-bp element located in the S component of the HCMV DNA[3].

1.5.6 Characteristics and functions of the late proteins

The L proteins are the last class of gene products expressed during HCMV replication. The L proteins have mainly structural roles and play a role in the assembly/packaging of the virion[3]. Their transcription begin more than 24 hpi and requires prior viral DNA replication[78]. L or γ proteins are divided in two subclasses: γ1 and γ2, depending on time of expression and sensitivity to viral DNA replication inhibitors. γ1 (leaky L) transcription occurs 24-36 hpi, and is reduced by such inhibitors. γ2 (true L) transcription occurs 24-48 hpi, and is strictly dependent on DNA replication.

Expression profiles of DNA microarrays for the whole HCMV genomes showed that the great majority of the transcriptionally active genes are L genes at 72 hpi (26% γ1 and 32% γ2, respectively)[72].

Transcriptional regulation of L genes and requirement of viral and/or cellular factors for their expression is still poorly understood. One of the most studied is the γ1 UL83 gene promoter, which is trans-activated by the combination of IE2-86 and IE1-72. The γ2 UL94 and UL99 promoters instead, require a minimal promoter element containing little more than the TATA box for full gene activation in late infection[3,66].

1.5.7 Virion assembly, maturation, and egress

Formation of HCMV capsids and packaging of viral DNA occur in the nucleus. Subsequently, nucleocapsids acquire a primary envelopment by budding at the nuclear membrane, and further mature through a de-envelopment/re-envelopment process in the cytoplasm before leaving the cell via an exocytotic-like pathway[3,94].

Nucleocapsid particles accumulated in inclusions that confer the typical 'owl's eye' appearance of the infected nucleus. The initial step is the interaction of pUL86 with the scaffolding AP pUL80.5 (the AP precursor) in the cytoplasm and the subsequent translocation into the nucleus, where oligomerization of these complexes catalyzed by AP leads to the formation of hexons and pentons that interact with pUL85-pUL46 complexes to form the B capsid precursor shell[23]. The subsequent association of capsid intermediates with pUL48.5 completes the formation of B capsids that are now ready to package viral DNA and, after insertion of viral genomes, remove AP[95]. During capsid formation, a series of proteolytic cleavages catalyzed by the assembling protein (pUL80a) leads to maturation of the UL80 precursor to assembling/APs and the dissociation of UL86 from AP[23].

Capsid are initially enwrapped through budding at the nuclear membrane (N), where they acquire a primary envelope derived from its inner leaflet[23]. They then cross the lumen, fuse with outer leaflet of the nuclear membrane or the endoplasmic reticulum membrane (ER) with which it is contiguous, lose their primary envelope, and move into the cytoplasm by vesicles (TE). Here, HCMV virion particles further mature by acquiring their tegument. The tegumented capsids then receive their definitive envelope by budding into vesicles of the Golgi apparatus (G)[39]. Both tegumentation and re-envelopment are driven by multiple specific protein-protein interactions to secure the integrity of the viral particle[94]. These mature particles are retained within the vesicles and transported to the cell surface via the Golgi network, which is enlarged due to the accumulation of nucleocapsids and DB. The Golgi alterations during the late replication stages create inclusions around the nucleus that result in its characteristics kidney-like appearance[3]. Progeny virus accumulates in the cytoplasm, and infectious virus is released into the extracellular compartment beginning at 72 hpi. In the very late stages, however, a substantial number of viral particles are still associated with the cell. A brief summary of the HCMV replication cycle is represented in Figure 3.

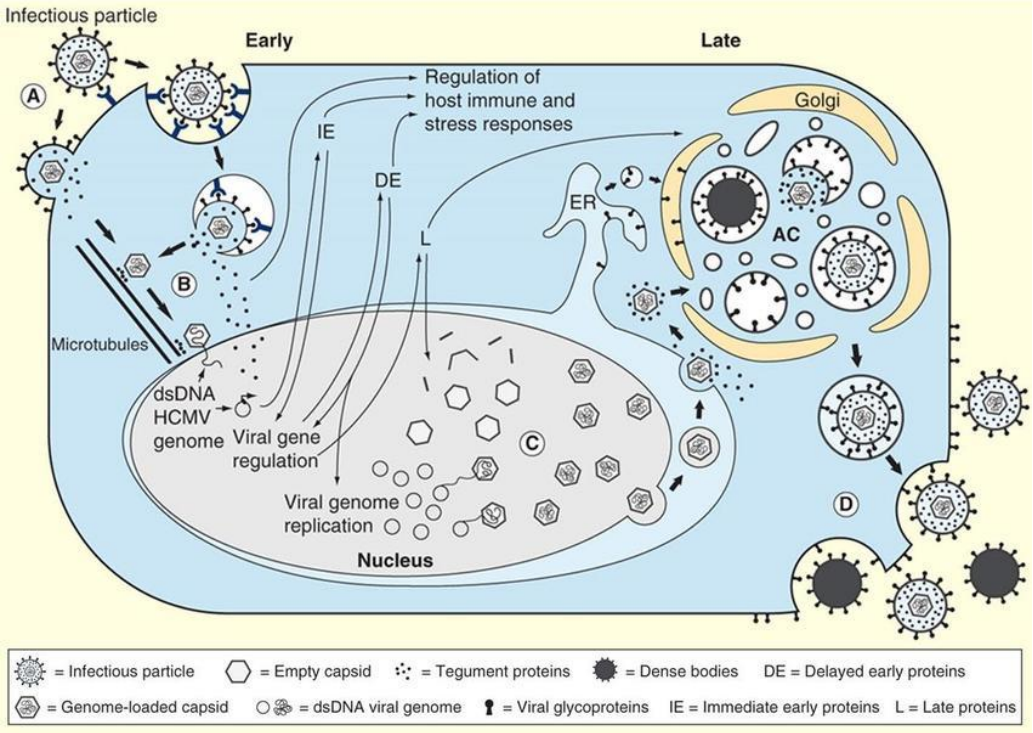

Figure 3

Schematic model of the HCMV lytic cycle in a cell[96].

1.6 The host immune response against HCMV infection

1.6.1 Innate immunity

CMV displays a broad tropism, being able to infect several cell types, such as endothelial cells, epithelial cells (including retinal cells), smooth muscle cells, fibroblasts, leukocytes and dendritic cells[58,97]. In healthy individuals, primary CMV infection initiates with replication in the epithelium of the upper alimentary, respiratory, or genitourinary tracts, even though it is not essential infection of the epithelial mucosa since infection is easily established by blood transfusion and transplantation[98]. Thus, transmission occurs through close contact with contaminated fluids, such as saliva, blood, urine, milk (breast-feeding), vaginal fluid (birth delivery), blood or organ-transplantation.

Leucocytes and vascular endothelial cells diligently support the systemic dissemination of the virus within the host, as virus encoded chemokines that may facilitate the spread from the initial site of replication by attracting neutrophils and monocytes[90,98,99].

Subsequently, the virus disseminates to monocytic cells of myeloid lineage, including monocytes and CD34+ cells, where it establishes latent infection. In these cells, gene expression is limited to the E genes leading to the hypothesis that bone marrow precursors are the chief reservoir for latent CMV infection and differentiation induces reactivation, productive infection and further dissemination[3].

As CMV enters cells to establish infection, the host recognizes the virions and activates several mechanisms and pathways of innate immune response, the first line of defense against CMV. These include inflammatory cytokines, type I Interferon (IFN) and upregulation of costimulatory molecules that are crucial for slowing the pathogen and subsequently priming a high-quality adaptive immune response[60]. This becomes particularly evident during the perinatal period because of the immaturity of adaptive immunity[23,100].

Detection of pathogens by the innate immune system involves a class of germline-encoded molecules termed pattern recognition receptors (PRRs).

Cellular sensors that detect and are activated by HCMV binding and entry are the Toll-like receptor 2 (TLR2) and CD14 receptor, that recognize CMV surface glycoproteins gB and gH, which leads to activation of an NF-κB-dependent signal transduction pathway[101]. Then, the intracellular dsDNA sensor Z-DNA binding protein 1 (ZBP1), TLR9, and cGAS[102,103]. The initial interferon response to HCMV infection is triggered when the cell detects viral attachment and entry, resulting in an early induction of IFN synthesis and secretion, in return HCMV has evolutionarily developed a suite of IFN countermeasures that occupy a significant portion of viral coding potential[104].

The activation of the innate immune system also includes recruitment of professional antigen-presenting cells (APCs), phagocytes and NK cells[60].

NK cells (usually expressing CD16 (FcγRIII) and CD56) function as important sentinels of the immune system and are considered a bridge between the innate and adaptive immune systems because of their ability to provide rapid cytotoxic function, through the production of IFN-γ, granzymes and perforins. In addition, NK cells most likely provide a cytokine milieu that supports and drives the subsequent maturation of adaptive immunity, in particular of T cells[105]. There is also increasingly compelling evidence that NK cells play a crucial role in host defense against viral infection[106,107]. Relatively little is known about the role of NK cells in the immune defense against human CMV, but the extensive immune evasion mechanisms that CMV encodes to prevent NK cell activation indicate their importance in the innate response to CMV[108]. Indeed, patients with rare genetic defects involving overexpression of an iKIR can have serious recurrent episodes of CMV disease[109]. NKs activation, proliferation, as well as destruction of virally infected cells depend upon complex arrangement of subsets expressing overlapping repertoires of invariant surface receptors, termed activating (aKIR) and inhibitory killer immunoglobulin-like receptor (iKIR) on NK cells determine NKs activation, proliferation, as well as destruction of virally infected cells[110]. One well established viral immune evasion strategy is shown by the viral pUL40, which is a ligand for the non-canonical HLA-I molecule HLA-E, upregulating its surface expression, and facilitating its binding with the inhibitory receptor CD94/NKG2A, therefore conferring resistance to NK-cell lysis[111].

Inflammasome. As already mentioned, the innate response is mediated by type I and III in IFNs and inflammatory cytokines, which very rapidly create an antiviral state in the host, thereby triggering the inflammatory response[112].

An important route of innate immunity relies on the inflammasome, a key component of cytosolic surveillance that controls activation of the proteolytic enzyme caspase-1 that leads to the production of interleukin 1-β (IL-1β) and IL-18[113]. The inflammasome consists of a multimeric protein complex commonly formed by sensor proteins known as PRRs, more in detail by the adaptor molecule known as apoptosis-associated speck-like proteins containing a C-terminal caspase recruitment domain (ASC or Pycard) that bridges cytosolic PRRs and pro-caspase-1, with the latter being converted from an inactive zymogen into an active protease, to catalyze the maturation of the pro-inflammatory cytokines interleukin 1-β (IL-1β) and IL-18 or the rapid inflammatory form of cell death called 'pyroptosis'. The activation of the inflammasome depends upon recognition of pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs).

Although a plethora of stimuli, such as nucleic acids, toxins, and metabolic products, trigger inflammasome assembly, the mechanism of inflammasome activation and inhibition in response to viral infections still remains partly understood[114].

The release of mature IL-1β upon HCMV infection has been reported in vitro[115]. Few reports showed inflammasome activation in HCMV-infected monocytes or macrophages.

Notably, AIM2 is essential for inflammasome activation in response to MCMV[116]. The AIM2 inflammasome has been described as a PRR that plays an important role in regulating immune responses to viral and bacterial pathogens[117–119]. AIM2 has two functional domains, the N-terminal pyrin domain and the C-terminal hematopoietic interferon-inducible nuclear (HIN) domain. AIM2 binds to DNA via the HIN domain, whereas the pyrin domain associates with the downstream adaptor molecule apoptosis-associated speck-like protein, containing a caspase recruitment domain (ASC) necessary to activate caspase-1[120]. Among HCMV proteins, the tegument protein pp65 seems to play a major role in immunomodulation and immune evasion[121,122]. Inregards, pp65 has been shown to downmodulate caspase-1 and IL-1 activation following the interaction of pp65 with AIM2[123]. In contrast, Li et al. showed that pp65 cannot bind to the AIM2-PY domain. Notably, a pp65 deletion mutant of HCMV was reportedly unable to activate the inflammasome to levels that were comparable to those with wild-type HCMV, as judged by caspase-1 cleavage. Moreover, they found any changes in caspase 1 cleavage during HCMV infection, suggesting that the canonical inflammasome assembly pathway was not essential for enhanced IL-1 production upon HCMV infection[124].

In our paper, we showed that the lack of tegument protein pp65 enhances the production of mature IL-1β, in part through the induction of DNA binding and the transcriptional activity of NF-κB. Lastly, we demonstrate that HCMV infection of HFFs triggers a non-canonical IL-1β activation pathway where caspase-8 promotes IL-1β maturation independently of caspase-1[125].

1.6.2 Intrinsic immunity

Intrinsic antiviral immunity refers to a form of innate immunity that directly restricts viral replication and assembly, thereby rendering a cell non-permissive to a specific class or species of viruses. Intrinsic immunity is conferred by restriction factors (RFs) that are largely preexisting in certain cell types, although these host factors can be further induced by virus infection. Intrinsic viral restriction factors recognize specific viral components, but unlike other pattern recognition receptors that inhibit viral infection indirectly by inducing interferons and other antiviral molecules, intrinsic antiviral factors block viral replication immediately and directly; thereby, providing a frontline defense against invaders. At the same time during the evolutionary 'arms race' for survival, viral proteins have successfully evolved to modulate or degrade RFs[126].

So far, several host proteins, including γ-interferon-inducible protein 16 (IFI16), viperin, nuclear domain 10 (ND10) complex, APOBEC3 cytidine deaminases, survival time associated PHD protein in ovarian cancer 1 (SPOC1) and myxovirus resistance B (MxB) have been proposed to counteract HCMV infection by restricting viral replication. Interestingly, HCMV, in its turn, has evolved effective countermeasures to resist them (Fig.4)[127].

	IFI16	Viperin	APOBEC3	SPOC1	MxB
RFs antiviral activities	• Interaction with Sp1 and HCMV pp65 to inhibit UL54 promoter • Interaction with cGAS and antiviral cytokine expression	• Inhibition of HCMV late gene expression	• Insertion of hypermutations into the HCMV genome through cytidine deamination	• Silencing of viral IE expression via epigenetic modifications	• Efficiency in restriction of herpesviruses of all three subfamilies, including HCMV, by targeting early viral gene expression
HCMV escape mechanisms	• Sequestration by pp65 for MIEP activation • Protection from proteasome degradation by pp65 • Delocalization upon phosphorylation by pUL97	• Delocalization by vMIA protein from the endoplasmic reticulum to the mitochondria to increase lipid synthesis and viral production	• Shaping the nucleotide composition of the HCMV genome	• Degradation during the late replication phase in a glycogen synthase kinase 3β (GSK-3β)-dependent manner	• It remains to be further established whether HCMV encodes a viral MxB antagonist or employs any other strategy to counteract MxB

Figure 4

The best-characterized host restriction factors in the defense against human cytomegalovirus and viral countermechanisms[127].

The IFN system comprises type I, II and III IFN, and involves hundreds of IFN-stimulated genes (ISGs). ISG expression can lead to the so-called restriction factors which directly inhibit a process essential to the production of virus progeny, or ISG expression can act indirectly by building up an elaborate antiviral network[128]. In this work the family of MxA and IFITs proteins has been considered for a potential new antiviral role against HCMV, due to the fact that these ISG are targeted by HCMV-induced citrullination, a phenomenon that strongly supports the viral replication. We hypothesized that HCMV-induced host citrullinated target and tackle MxA and IFITs protein in order to suppress their antiviral function.

Myxovirus resistance proteins are known as key players in the innate immune response to viral infections. Humans express two paralogous *Mx* genes, *MX1* and *MX2*, encoding the MxA and MxB proteins, under strict IFN type I and type III control. Both are dynamin-like GTPases containing a G domain responsible for GTP hydrolysis and a stalk region involved in oligomerization[129–131]. Only recently, two different groups analyzed the activity of MxB against the three herpesvirus subfamilies and showed that MxB serves as a broadly acting intracellular restriction factor[132,133]. For many years MxB was not considered to play any role against viral infections, until in 2013 Liu et al. showed that MxB inhibits HIV infection by reducing DNA integration[134]. After that moment, the search after MxB functions has started.

On contrary, the antiviral activity of the cytoplasmic human MxA protein is broad, against a wide range of RNA and either some DNA viruses, irrespective of their subcellular site of replication. It is presently not clear how MxA can inhibit such a diverse group of viruses and whether there is a common denominator governing Mx sensitivity, considering that it is located in the cytoplasm in association with membranes of the smooth endoplasmic reticulum[135–137].

The family of IFIT (interferon-induced protein with tetratricopeptide repeats 1) includes four known human members (IFIT1, IFIT1, IFIT3 and IFIT5), which expression is induced by type I IFN α/β and they are able to form a multiprotein complex, perhaps to antagonize viruses by sequestering specific viral nucleic acids[138]. IFIT proteins inhibit the replication of multiple families of viruses through distinct mechanisms of action (Fig.5). For example, a recent study showed that human IFIT1 can sense RNA viruses having uncapped 5'triphosphorylated RNA that will be sequestered by the actively replicating pool. Knocking down of IFIT1, IFIT2 and IFIT3 showed increased rate of the Rift Valley fever virus (RVFV), vesicular stomatitis virus (VSV), and influenza A[139]. IFIT1 has also been shown to bind preferably to 2'-O-unmethylated RNA (uncapped), inhibiting translation of viral RNA. Thus, viruses lacking 2'-O-methylation, such as

West Nile virus (WNV) or Japanese Encephalitis Virus (JEV) could replicate only in cells lacking IFIT1[140,141]. IFIT proteins don't have a nucleic acid-binding domain, but mediates the binding to nucleic acid sequences with a highly charged carboxy-terminal groove, where the Arginine 187 seems to be necessary according to Pichlmair et al.[139]. In addition, IFIT1 has been found to suppress internal ribosome entry site (IRES)-dependent viral RNA translation during Hepatitis C virus (HCV) infection[142]. IFIT1 can also bind a viral protein IE1, a viral helicase from human papillomavirus (HPV), which is sequestered in the cytoplasm to prevent replication[143,144]. In 2015 Zhang et al. showed for the first time that IFIT1 has important antiviral replication effect against HCMV in fetal astrocytes[145]. More recently, in 2019 Li and Swaminathan[146] have shown that human IFIT1, IFIT2 and IFIT3 proteins can suppress lytic replication of another member of the herpesvirus family, the KSHV, but the mechanism of action is still unknown.

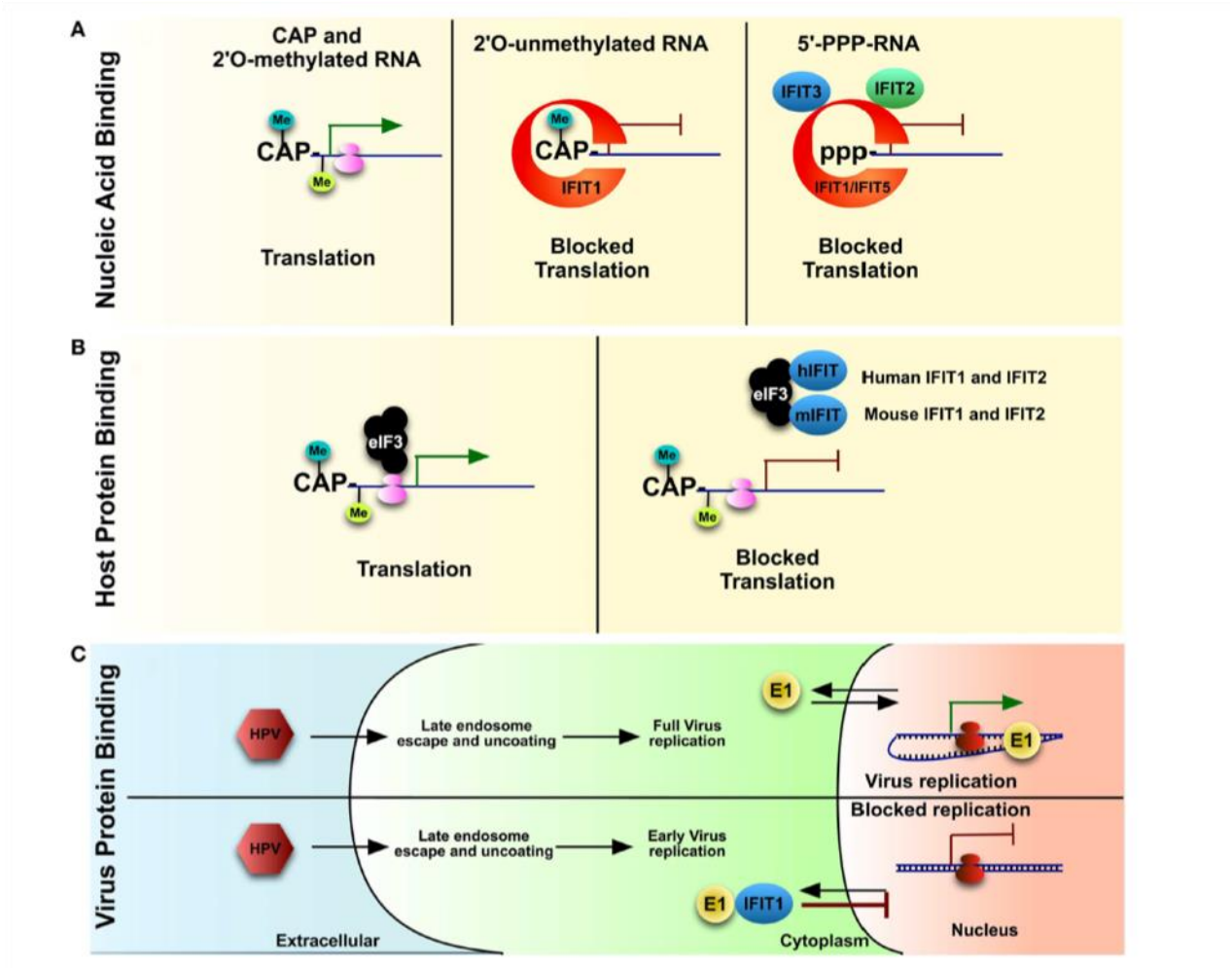

Figure 5

IFITs, once upregulated due to IFN signaling, play various roles in blocking virus and host protein translocation[147].

1.6.3 Adaptive immunity

Following establishment of primary CMV infection, a complete and long-lasting adaptive immune response is necessary in healthy individuals, first to control CMV infection and secondly is vital to maintain CMV latency and prevent productive (lytic) infection. Since severe infection is usually restricted to individuals with impaired cell-mediated immunity, it is evident that this arm of the immune response provides the most protection[148]. The adaptive immune response to CMV engages both humoral and cellular immunity[149–151].

Humoral response. During CMV primary infection, antibodies specific for multiple CMV proteins are elicited in the host. At first IgM antibodies are induced and persist for 3-4 months, followed by IgG antibodies that persist for life[148]. They recognize structural tegument proteins (e.g., pp65 and pp150), envelope glycoproteins (predominantly gB and gH and gH/gL multimeric complexes), and nonstructural proteins such as the IE1 protein[97,152]. The role of antibodies for protection against and control of CMV has been debated. However, studies indicated that humoral immunity is crucial in restricting viral dissemination and probably contribute to minimizing the clinical manifestations of the disease[152]. It has been shown that seronegative transplant recipients who receive the organ by seropositive donors, if preimmunized with high-titer anti-CMV Igs, display a less severe primary infection[153]. Several *in vivo* experiments have also supported the hypothesis that humoral response is important for CMV control[154,155].

Cellular response. The size of the CMV-specific T-cell response is the most striking aspect of the dynamic, life-long interaction between the host and CMV. The immune system commits more resources to controlling HCMV than any other virus, indeed healthy seropositive individuals often have more than 1% of their blood peripheral T cells specific for one antigen of HCMV[156]. T cells are essential to restrain CMV viral replication and prevent disease, but do not eliminate the virus or preclude transmission, in particular cytotoxic T lymphocyte (CTL)[157–159]. Interestingly, broadly targeted CMV specific T cells dominate the memory compartments of exposed subjects, approximately 10% of both the CD4+ and CD8+ memory compartments in peripheral blood[156]. The extent to which high frequencies of CMV-specific T cells are required for viral control remains unclear.

The viral proteins targeted by CTLs include structural and non structural forms: UL83 (pp65) and UL123 (IE) are the chief targets, but several other ORFs were also at the top of the recognition hierarchy for both CD4+ and CD8+ T-cell responses[3,156].

Since the time course of the appearance of CMV-specific adaptive immune responses is difficult to follow in asymptomatic healthy subjects, the majority of the observations on CMV infection dynamics have been carried out in CMV-naive recipients of kidney transplant from CMV-seropositive donors.

Generally, first CD4+ T cells specific for the virus start to circulate 1 week after infection, and synthesize Th1 cytokines (e.g., IFN-γ, tumor necrosis factor α (TNF-α))[160,161]. Subsequently, CMV-specific CD8+ T cells appear in the peripheral blood. The so-called CTLs that have a direct role in the resolution of the infection express both perforin and granzyme B, and are capable of lysing CMV peptide-presenting target cells[162,163]. Passive administration of autologous HCMV specific CD8+ CTLs at set intervals after bone marrow transplant (BMT) to seronegative recipients of marrow from seropositive donors generated a vigorous CMV-specific CTL response without onset of viremia or CMV disease[164].

Following recovery from CMV infection, resting virus-specific CD8+ T cells express surface markers characteristic of memory T cells.

Importantly, early and late virus-specific T CD8+ cells display a Th1 cytokine signature with predominant and constitutive production of IFN-γ, and they accumulate during latency.

These CD8+ effector T cells retain the capacity to almost instantaneously secrete cytokines upon T-cell receptor ligation, essential to antagonize viral replication upon reactivation of latent virus [162,165,166].

1.7 HCMV pathogenesis

1.7.1 Congenital infections

Maternal transmission to the fetus of a new or reactivated latent infection may occur at any gestation, leading to congenital CMV. About 20,000-40,000 infants per year in the United States are born with congenital CMV infection, with a corresponding incidence of 0.6 - 0.7% of all deliveries of the developed world, making CMV the most common congenital viral infection[167–169].

Infection may result in neurodevelopmental delay, foetal or neonatal death, and most frequently sensorineural hearing loss and other CNS abnormalities, which is particularly severe when primary maternal infection occurs during the first 3 months of pregnancy, when organs are developing and neuronal migration is occurring[23,170,171]. During gestation, anti-CMV antibodies in CMV-seropositive pregnant women play an important role in preventing congenital infection of the fetus[152]. Seronegative pregnant women carry an approximate 40% risk of CMV transmission to the fetus.

Although primary infection during pregnancy as a source of fetal infection has a high transmission rate to the fetus when the mother gets infected, even higher rates are observed in populations with higher rates of maternal seropositivity, suggesting that most congenital infections are caused by reactivation of latent virus[172,173]. Among the congenitally infected newborn, 5-10% display cytomegalic inclusion disease, whose symptoms include intrauterine growth retardation, jaundice, hepatosplenomegaly, thrombocytopenia, petechiae, chorioretinitis, and hepatitis, along with central nervous system (CNS) involvement (microcephaly, encephalitis, seizures, and focal neurological signs). While the neurological damage is permanent and accounts for the long-term morbidity and poor prognosis of cytomegalovirus inclusion disease, most of the non-CNS manifestations are self-limiting and resolve without therapy in the vast majority of cases [172].

In 11–20%, damage caused by the virus leads to such a severe life-threatening organ dysfunction that the patient dies during infancy. Long-term follow-up shows that 15% of the symptomatic infected infants at birth (90–95% are asymptomatic) may develop hearing defects or impaired intellectual performance.

1.7.2 Immunocompromised hosts

HCMV is a significant opportunistic pathogen in immunocompromised patients.

CMV infection or reactivation is a frequent cause of morbidity and mortality in immunocompromised transplant recipients. Antirejection therapy is administered to limit T-cell-mediated graft rejection and both suppress antiviral immunity, targeting both CD8+ and CD4+ critical for CMV control. This deficit leads to CMV reactivation, uncontrolled replication and dissemination leading to life-threatening end-organ disease, termed CMV disease (pneumonitis, colitis and hepatitis)[174,175]. At this point, patients are normally treated with antiviral chemotherapy that may later lead to a late-onset CMV disease associated with drug resistance during long-lasting therapies.

HCMV disease is more difficult to treat in BMT recipients and AIDS patients with low CD4+ T-cell counts compared with solid organ transplant recipients, and HCMV pneumonia has a high mortality rate, despite the recent introduction of specific antiviral drugs[176]. HCMV is initially localized in the transplanted organ, i.e., hepatitis occurs generally in liver transplant recipients, pancreatitis in pancreas transplant recipients, but then spreads throughout the gastrointestinal tract and to the retina, skin, endometrium, lungs, and CNS[177].

Lastly, an immunosuppressive syndrome often related to HCMV infection in the late post-transplant period is characterized by superinfection with bacteria, fungi, and protozoa, perhaps due to disturbance of both the humoral and cellular immune response by HCMV[178].

1.7.3 The role of HCMV in the development of other diseases

As mentioned earlier, HCMV has emerged as an important human pathogen in immunocompromised patients, such as AIDS patients and organ-transplant recipients. In such patients, HCMV-mediated disease has highlighted the possible role of this virus in the development of other diseases, such as inflammatory diseases (autoimmune and vascular diseases) and cancer. It is not known whether under specific conditions HCMV may possess a causative role or be an epiphenomenon in these diseases, meaning a bystanding factor that promote the disease and/or aggravate the disease severity[179].

HCMV and autoimmune diseases. An AID is characterized by an aberrant and chronic immune response against self-antigens, generally mediated by the generation of autoantibodies and/or cellular response. It is well known that not only genetic predisposition is sufficient to develop an AID, but also environmental factors represent intriguing co-factors of these multifactorial diseases. HCMV has been implicated in type 1 diabetes (T1D), in rheumatoid arthritis (RA), in Sjögren's syndrome, multiple sclerosis (MS), systemic sclerosis (SSc), in systemic lupus erythematosus (SLE) and also in inflammatory bowel disease (IBD).

According to the several studies collected and examined in a study by Halenius and Hengel, there is no evidence that HCMV plays a role for the onset of T1D and MS. However, concerning SLE and SSc based on several studies that report an association, it cannot be ruled out that HCMV plays an active role in the induction of SLE depending on largely unknown genetic factors[180–183]. One fascinating evidence was reported using lupus-prone murine models, where the injection with pp65 peptides induce early onset of autoantibody and glomerulonephritis, while in BALB/c mice led to the development of autoimmune-like phenomena that resemble SLE[184,185].

Several features of HCMV including its extensive manipulation of adaptive and innate immune functions, the very large coding capacity, its lytic replication in multiple tissues both locally and systemically, its lifelong persistence during subsequent phases of latency and reactivation, and its ubiquitous prevalence in human populations readily explain why HCMV was frequently linked with AID but also with further acquired disorders like arteriosclerosis and vascular disease, immune aging, and certain types of tumors[13,49,159,186–191].

Regarding the mechanisms, viral infections may trigger or elicit an autoimmune response by two distinct phenomena: **molecular mimicry** and **bystander activation**[192–195]**.** A molecular mimicking event occurs when pathogenic foreign epitopes are highly similar to host determinants leading to cross-activation of the immune system causing a self-attack. For bystander activation, it is hypothesized that during infection infected tissue and activated antigen presenting cells (APC) will attract immune cells producing high levels of cytokines and chemokines, which lower the threshold

for immune activation for a loss of tolerance. In support of molecular mimicry's hypothesis, it was shown that CD13-specific autoantibodies were present in the sera of BMT recipients with HCMV disease, but not in patients without any sign of HCMV infection. CD13 is an aminopeptidase and a receptor for HCMV entry present on all cells susceptible to HCMV, as well as in infectious particles[196]. In some way CD13 becomes immunogenic in BMT patients (Initiator factor).
It was suggested that latent HCMV infection is reactivated in patients with AIDs and that active viral infection in the tissues affected may aggravate the inflammation (epiphenomenon).
The chronic inflammation associated with AIDs provides an ideal microenvironment for reactivation of latent HCMV in inflammatory macrophages. Human cytomegalovirus can replicate in macrophages which differentiate from monocytes stimulated by cytokines such as IFN-γ and TNF-α[197,198]. These cytokines are often produced by activated T cells. TNF-α activates the HCMV IE promoter in myeloid cells, thereby enhancing replication of the virus, but this cytokine is not sufficient to reactivate latent HCMV[199–204]. In fibroblasts, HCMV infection induces translocation of NF- κB, which can interact with the HCMV IE promoter within hours[205,206]. Thus, HCMV-induced TNF-α production may enhance replication of the virus in certain cells through secondary activation of NF-κB[207]. As a matter of fact, active HCMV infection is detected in the majority of patients with ulcerative colitis and Crohn's disease, supporting the hypothesis[208].

HCMV and vascular diseases. Coronary atherosclerotic heart disease, also known as coronary heart disease (CHD), involves the coronary and other circulations, and is normally caused by atherosclerosis (AS), via vascular stenosis and/or obstruction, resulting in myocardial ischemia and hypoxia and even death. AS is prevalent in the elderly: in Western society it is the primary cause of death[209]. The development of AS is closely related to inflammatory reactions and the immune response, endothelial injury, lipid deposition, and coagulation thrombosis[210].
In recent years, although traditional risk factors for the development of AS, such as high blood lipids, high blood pressure, smoking, obesity, and diabetes, have been effectively reduced, the incidence of AS is still high, suggesting that other factors are involved[211–217]. In addition, 30–50% of patients with AS lack these classic risk factors. Epidemiological studies have shown that pathogens such as Chlamydia, and Cytomegalovirus (CMV) are involved in the occurrence and development of AS[218–221]. An association between CMV infection and AS was first reported in 1987, where Adam showed that the infection rate of HCMV and antibody titer were higher in AS patients compared to controls[222]. In 1983 Melnick et al. already showed that plaques of atherosclerotic patients were positive for HCMV antigens, while surrounding tissues weren't. In recent years, many serological and molecular-biological studies have shown that human CMV (HCMV) infection of endothelial cells (ECs) plays

an important role in the development of AS[223–225]. Fabricant et al. were the first to demonstrate that atherosclerosis in chickens can be caused by infection with Marek's disease virus, a herpes virus that infects fowl[226]. Further studies in mice showed that CMV accelerates atherosclerosis[227].

A proposed role of HCMV in the pathogenesis of atherosclerosis involves the reactivation of a latent HCMV infection. First of all, HCMV infects ECs leading to cellular injury and metabolic changes with release of growth actors, chemokines and cytokines, which promote monocytes and macrophages recruitment and platelet aggregation[228]. Interestingly, the viral protein US28 is a chemokine receptor homologue and has been shown to induce smooth muscle cells (SMCs) migration as well[229]. Viruses from ECs then infect SMCs and the latent or persistent infection leads to proliferation and accumulation of cholesterol and cholesterol esters leading to vascular inflammation and damage through uptake of low-density lipoproteins by smooth cells, neointimal formation (thickened arterial layer via cell migration and proliferation), and narrowing of the vessel lumen[230]. In cases of low immunity such as in immunocompromised patients, latent infection is repeatedly activated, resulting in repeated damage to the arterial wall. As a matter of fact, CMV is associated with ischemic heart disease (IHD) among organ transplant recipients. Gkrania-Klotsas et al. showed that CMV IgG antibody levels are associated with incident IHD compared to seronegativity in the population-based EPIC-Norfolk study[231]. Infection with CMV, particularly in susceptible disease states such as diabetes, may be an important risk factor for CHD and there is at least a modest association between CMV and asymptomatic carotid wall thickening, consistent with early AS[232,233].

On the other side, few epidemiological studies haven't found any association between CMV and AS. One of them was done in Germany, they collected 312 patients with more than 50% with coronary artery stenosis and 479 healthy controls. There was no significant difference in HCMV seropositivity or inflammatory marker related to the infection[234].

It is clear that future research should focus on the underlying diseases caused by the indirect effects of the virus, and HCMV-related AS is one such disease. Although considerable experimental and epidemiological evidence suggests that HCMV is associated with AS, the molecular mechanisms linking the two are unclear. This will be helpful for developing new drugs to prevent or delay AS formation. Interestingly, statin treatment, that inhibits the enzyme HMG-CoA reductase which plays a central role in the production of cholesterol, has recently been reported to inhibit the HCMV replication in endothelial cells[235].

HCMV and cancer. HCMV has been found in patients with colon cancer, malignant glioma, EBV-negative Hodgkin's disease lymphoma, cervix cancer, prostatic intraepithelial neoplasia and prostatic

carcinoma[236–240]. Interestingly, active infection has been detected in several of these tumors, but not in the noncancer sorrounding cells. HCMV is not able to induce directly transformation of normal human cells, but can only promote an oncogenic process (**oncomodulation**)[14].

After all, HCMV is well known for its ability to interfere with several key signaling pathways, such as inhibition of apoptosis, modulation of the host immune, and therefore may support survival, angiogenesis, migration and invasiveness[241]. For example, IE86 has been shown to interact with p53, a cell cycle regulator involved in cell cycle arrest, response to DNA damage and apoptosis, indeed abolishing its ability to transcriptionally activate a reporter gene[242]. The ability of HCMV to inhibit apoptosis is well known: the viral gene UL37 and viral induction of Bcl-2, an anti-apoptotic gene, protect Hela cells and neuroblastoma cells against apoptosis induced by chemotherapeutic drugs[243,244]. HCMV infection may alter the pattern of surface-expression adhesion molecules promoting tumor migration and invasiveness. For example, neuroblastoma cells infected with HCMV exhibit augmented invasiveness due to expression of VLA-5 integrin a1b5 together with downregulation of neural cell adhesion molecule (CD56)[245]. Infection of cells with HCMV also leads to upregulation of expression of ICAM-1, CD11b, Sialyl–Lewis antigens, and in addition, the virus encodes for four putative chemokine receptors[246,247].

However, studying the linkage between HCMV infection and tumor has not been an easy task. Indeed, after subculturing the virus containing tumour tissue HCMV could not be detected[14].

Eventually, HCMV may participate in the development of several aforementioned pathologies. Thus, the drugs now available for treatment of HCMV disease (e.g. GCV, acyclovir and foscarnet), may also prove to be useful in the treatment of other, more widespread diseases.

1.8 Treatment

1.8.1 Antiviral drugs

Successful control of viral infections during pregnancy and in the newborn period is essential for reducing early and late morbidity and mortality. Five compounds are currently licensed to treat established HCMV infections: ganciclovir (GCV), its oral prodrug valganciclovir (VGCV), foscarnet (FOS), cidofovir (CDV), and fomivirsen (Table 2)[248].

GCV is a competitive inhibitor of viral DNA polymerase (UL54), and its antiviral activity requires monophosphorylation in the infected cell by the phosphotransferase encoded by the UL97 gene of HCMV[249], followed by diphosphorylation by cellular kinases.

It is administered by intravenous infusion, and is usually the front-line drug for the treatment of HCMV infections. An oral formulation that is effective for prophylaxis in immunocompromised patients has also been approved[250].

PFA is a noncompetitive inhibitor of the pyrophosphatebinding site of HCMV DNA polymerase, which does not require prior activation by a virally encoded enzyme. It is administered intravenously as an alternative to GCV in the event of GCV resistance or severe side-effects, although its application is limited by nephrotoxicity and disturbance of the electrolyte balance[251,252].

CDV is a competitive inhibitor of the HCMV DNA polymerase, and has been approved for the treatment of HCMV retinitis[253]. One of its advantages compared with GCV and PFA is its long intracellular half-life [254,255].

To date, GCV, CDV and PFA have been licensed for serious or life-threatening HCMV infections in immunocompromised individuals. While, therapies consisting of intravenous GCV or oral VGCV for 6 weeks are the only accepted treatment options for patients with symptomatic congenital HCMV disease involving the CNS.

However, despite encouraging clinical outcomes, the use of these drugs has been hampered by major hematologic adverse effects (e.g. neutropenia, thrombocytopenia, and anemia) along with their long-term toxicity, low potency, and poor bioavailability[19,256].

Since all these compounds, with the exception of fomivirsen, target, either directly or indirectly, the HCMV DNA polymerase, the emergence of drug-resistant viral strains often due to mutations in UL97 and/or UL54, has increasingly become a major problem for disease management[257,258]. Thus, there is an unmet and urgent medical need for new anti-HCMV drugs with novel mechanisms of action able to overcome antiviral drug resistance and adverse effects displayed by currently available compounds. Interestingly, an antisense oligonucleotide against the HCMV IE2 mRNA (ISIS2922, fomivirsen) has also been developed[259,260] for intravitreal application in patients with retinitis who do not respond to conventional management[261].

A promising new class of anti-HCMV compounds is currently on clinical trial (Table 2). Maribavir is a riboside analogue, but with a novel mechanism of action. It inhibits the UL97 enzyme, which breaks down the nuclear lamina to allow egress of virions into the cytoplasm and also been shown to interfere with viral DNA synthesis. Clinical trials with Maribavir have not been conclusive so far and the compound is still under examination at a higher dose, as pre-emptive therapy. BAY 38-4766 (Bayer Pharmaceuticals), represents a novel class of nonnucleoside antiviral agents. It is a highly selective inhibitor of CMV *in vitro*[262]. Antiviral activity of BAY 38-4766 results from inhibition of DNA maturation, most likely targeting UL89 and UL56 genes, which encode subunits of the viral terminase[263]. Interestingly, in a guinea model after infection and treatment, measurable amounts of

drug were detected in fetal blood, indicating that the compound crosses the placenta in pregnant guinea pigs[264]. BAY 38-4766 entered clinical development and showed a favorable safety profile in healthy male volunteers at single oral doses up to 2000 mg. However, no recent reports have revealed the current status of clinical development of this compound or related compounds in the series. GW275175X is a novel benzimidazole riboside class of CMV inhibitors[265]. It has been shown to block the maturational cleavage of high molecular weight CMV DNA by interaction with pUL56 and pUL89, the two subunits of the viral terminase complex[265]. GW275175X was advanced through a Phase 1 single-escalating dose trial of safety, tolerability and pharmacokinetics, but was then shelved in favor of the advancement of maribavir. The clinical potential of this early candidate is yet to be determined. Brincidofovir, a lipid prodrug of cidofovir that is rapidly taken up into cells and is not a substrate for the oxyanion transporter in the kidney, therefore kidney damage should be reduced although gastrointestinal toxicity is dose limiting[266]. Letermovir is a promising antiviral approach, it inhibits the terminase enzyme complex that normally cleaves concatameric DNA into unit lengths as it is packaged into the virion[267].

An important therapeutic approach against viruses would be the targeting of host factors necessary for a productive infection to prevent the development of resistance, which is an important problematic in RNA viruses due to high rates of mutation, but also in DNA viruses such as CMV.

There are two different strategies: prophylaxis or pre-emptive treatment. In prophylaxis, the antiviral drug is administered before active HCMV infection is detected to prevent its occurrence. With pre-emptive therapy, the antiviral treatment is initiated when HCMV positivity is detected in the blood or broncoalveolar lavage fluid using sensitive methods, such as PCR, nucleic acid sequence-based amplification, and tests for viral antigen. While the benefits of prophylactic therapy are controversial, the advantages for the pre-emptive one include the targeting of antiviral therapy to those most at risk for future disease, reducing the number of patients exposed to antiviral toxicity, lowering the risk of drug resistance, and maximizing the cost:benefit ratio[267–270].

Compound name	Drug Class	Year of Approval	Approved Indication
Gancyclovir	Nucleoside analogue	1989	CMV retinitis in AIDS patients and CMV disease in transplant recipients

Valgancyclovir	Nucleoside analogue	2001,2003,2009	CMV retinitis in AIDS patients and CMV disease in transplant recipients, also at pediatric age (4 months to 16 years)
Foscarnet	Pyrophosphate analogue	1991	CMV retinitis in AIDS patients
Cidofovir	Acyclic nucleoside analogue	1996	CMV retinitis in AIDS patients
Maribavir	Nucleoside analogue	Clinical trial	
Letermovir	Antisense oligonucleotide	Clinical trial	
BAY-38-4766	Non-nucleoside agent	Clinical trial	
GW275175X	Benzimidazole riboside	Clinical trial	
Cidofovir Esthers	Acyclic nucleoside analogue	Clinical trial	

Table 2 Scheme of marketed anti-CMV drug and in clinical development with drug class, year of approval and approved indication.

1.8.2 Prophylaxis

Prophylaxis with antiviral agents and pre-emptive therapy have proved useful in transplant patients. Development of a vaccine to prevent congenital HCMV infection is a priority concern, since the virus is a leading cause of CNS damage in children[271] . The potential benefit of vaccine-induced immunity has been estimated at 40-fold reduction with respect to intrauterine infection and 25- to 30-fold reduction with respect to decrease in CNS damage[272]. However, no vaccine is available and live-virus vaccines have not obtained official approval[273,274]. Both humoral and cell-mediated immunity are needed to prevent HCMV disease.

CMV vaccines has been generated using attenuated or chimeric viruses, DB, recombinant proteins, DNA, peptides and/or viral vectors[275]: in detail, attenuated live virus vaccines (Towne strain), recombinant live vaccines using chimeric viruses comprising genomic portions of the virulent strain Toledo and the attenuated strain Towne, canarypox-HCMV recombinants expressing gB and pp65, vaccination with HCMV DB, subunit gB vaccine; pp65 peptide-based vaccine, and DNA vaccines encoding the gB and pp65 proteins.

Many studies indicate that gB and pp65 are absolutely necessary vaccine components to secure the induction of neutralizing antibodies and CTL responses[276–280]. This is because pp65 is expressed by CMV-infected cells at early and late stages of infection.

The attenuated Towne strain, the recombinant gB, and the canarypox gB and pp65 vaccines already have been tested in clinical trials.

The attenuated Towne vaccine has been tested both in healthy volunteers and in transplant recipients[281–284]. It induced neutralizing antibodies and a CTL response and reduced HCMV disease in seronegative renal transplant recipients, but did not prevent infection. However, there was no evidence of its efficacy in a placebo-controlled trial in seronegative mothers of HCMV-infected children. To circumvent the risk of revertance, poxviruses with limited potential for replication in humans (canarypox) have been tested as vehicles for the expression of recombinant gB and pp65[285,286]. A subunit vaccine composed of a modified gB protein was evaluated after combination with a new powerful adjuvant, MF59, based on an oil-in-water emulsion of squalene. Results of a trial of 46 seronegative adults established safety, immunogenicity, and optimal antigen dose[287]. Whether the neutralizing antibodies elicited by this vaccine will be sufficient to prevent recurrence or primary HCMV infection is still an open question[288]. Other promising preclinical approaches include vaccination with HCMV DB and administration of DNA vaccine encoding viral immunogenic proteins to elicit a humoral and a CTL response[289,290].

2 Citrullination

2.1 Basic insights into the process of citrullination

Citrullination, also called deimination, is a process where the imine group of a peptidylarginine residue is enzymatically substituted by a carbonyl group, thereby forming the amino acid citrulline, a non-genetically encoded amino acid (Fig. 6), which leads to a mass increase of 0.984 Da and the loss of one positive charge *per* converted arginine residue. This loss of positive charge affects the acidity of the amino acid residue, changing the isoelectric point from 11.41 for arginine to 5.91 for citrulline[291].

This small alteration affects the charge distribution, the isoelectric point, the protein folding, that influence intra- and inter-molecular interactions resulting in changes of conformation and stability. The subsequent conformational changes may promote the formation of new motifs for protein (un)binding, may generate neoepitopes, and possibly alter the function and half-life of the modified proteins[292].

A key feature of citrullination is that it makes proteins more prone to degradation. When MBP is hypercitrullinated, it is degraded faster by proteases such as cathepsin D due to its more open conformation[293].

Today citrullination is considered an irreversible process.

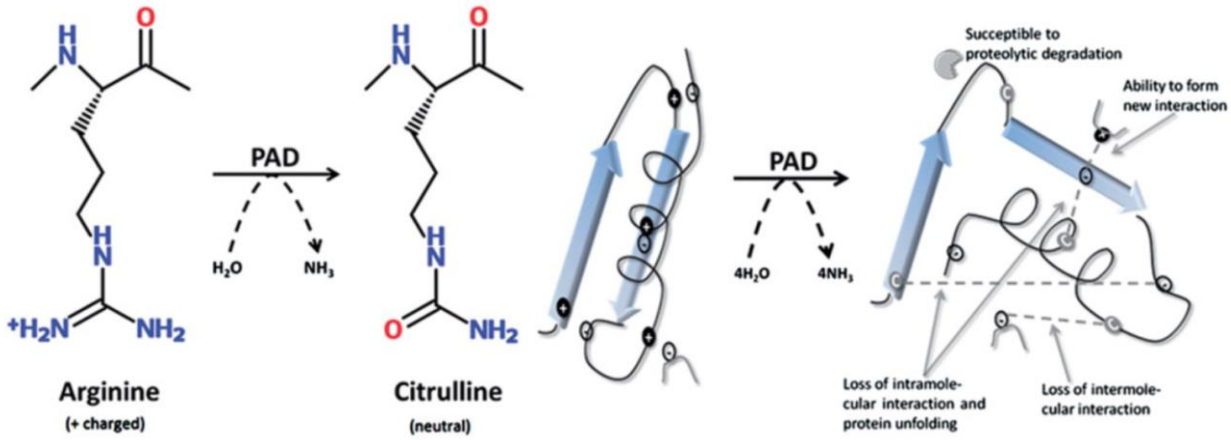

Figure 6

Citrullination of peptide-bound arginines by peptidyl arginine deiminase (PAD). PAD converts arginine into citrulline leading to the loss of one positive charge. PADs are Ca2+ -dependent enzymes[294].

In mammals, particularly in humans, there are five highly conserved isoforms of PAD enzymes. The genes encoding PAD1-4 and PAD6 are located in a single cluster on chromosome 1. Thus, the different isoforms are thought to be the result of multiple duplications of the same gene before the divergence of the mammalian species. Each enzyme consists of two immunoglobulin-like domains at the N-terminus and of an α/β propeller fold at the C-terminus, the latter containing the catalytic domain (Fig.7). They share 50–55% sequence identity, with greater similarity in the C-terminal two-thirds of the proteins. Importantly, while all PADs have been detected in the cytoplasm, PAD2 and PAD4 have also been found in the nucleus where they regulate deimination of histones leading to chromatin structure changes[295]. The presence of an N-terminal nuclear localization signal (NLS) in PAD4 confirms its translocation to the nucleus, while PAD2 may preferably take advantage of shuffle proteins to translocate[296,297].

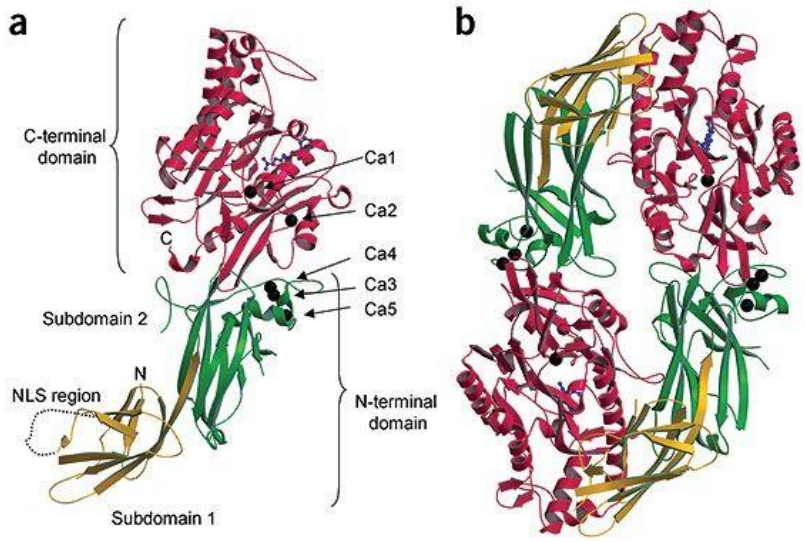

Figure 7

(**a**) **Ribbon representation of the monomeric form of the substrate complex of PAD4.** Five Ca^{2+} ions (Ca1–Ca5) are black balls, and the substrate, benzoyl-L-arginine amide, is a dark blue ball-and-stick model. Subdomains 1 and 2 and the C-terminal domain are yellow, green and red, respectively. The putative nuclear localization signal (NLS) region is shown by a dotted line. (**b**) Ribbon representation of the dimeric form of the substrate complex. A crystallographic two-fold axis runs vertically through the center of the dimer[298].

The PAD enzymes exhibit tissue-specific expression patterns. PAD1 is expressed in epidermis and uterus, PAD2 in muscle cells, spleen, salivary glands, CNS, and macrophages, PAD3 in hair follicles, tongue and nails, PAD4 in white blood cells of the immune system, and PAD6 in oocytes and embryonal tissues. Moreover, each enzyme displays distinct substrate specificity: PAD2 efficiently citrullinates α/β actin, whereas PAD4, among other substrates, citrullinates histone 3 (H3) during the formation of neutrophil extracellular traps (NETs)[299].

For the catalysis of arginine into citrulline, the presence of calcium ions is required. In particular, PAD cooperatively binds five calcium (Ca2+) ions in highly conserved binding sites of the subdomain 2 and the catalytic domain converting the enzyme into its active form (Fig.7)[298].

The sufficient Ca2+ concentration for *in vitro* PAD activity is 10-100 μM. At physiological conditions, intracellular Ca2+ levels are quite low (100 nM), suggesting that regulatory proteins may stabilize PAD *in vivo* lowering its Ca2+-based activation threshold[300]. The amino acids involved on the deimination process by PAD4 are aspartate 350, histidine 471, aspartate 473 and cysteine 645. The latter, in particular, mediates the initial nucleophilic attack on the guanidium carbon of arginine (Fig. 8). Arginine is oriented towards cysteine mediated by the two aspartate residues, whereas histidine fulfills its action playing as an acid and a base[291]. Finally, the intermediate is hydrolyzed to citrulline. Interestingly, PAD enzymes has been found only in a prokaryote named *Porphyromonas Gingivalis*, that causes periodontitis in humans. This enzyme could represent the match between bacterial infection and RA pathogenesis[301].

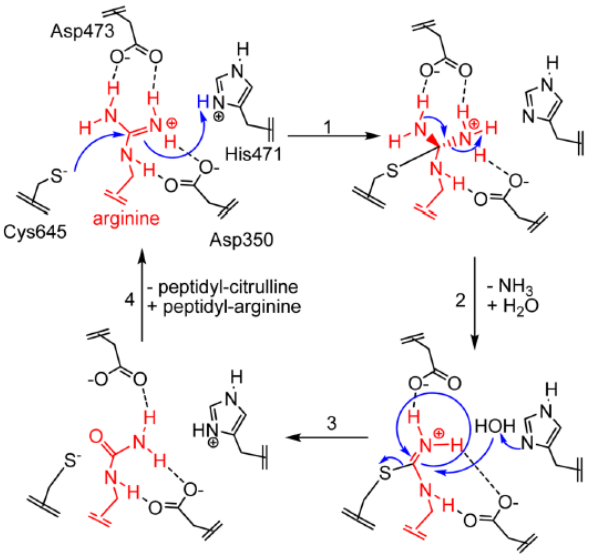

Figure 8

Catalytic mechanism of citrullination.
The guanidium group of the arginine (red) is oriented towards cysteine 645 that mediates the initial nucleophilic attack (blue arrow). As histidine 471 donates a proton (blue arrow), a tetrahedral intermediate is formed that is converted to a S-alkylthiouronium intermediate after the release of NH3. Subsequent hydrolysis of this intermediate generates citrulline (blue arrow)[302].

Another feature of the mechanism of action of PAD4 is dimerization. Even if PAD4 is able to citrullinate as a monomer, dimerization increases its enzymatic activity and moreover is necessary for Ca2+-binding cooperativity[303].

Under physiological conditions, citrullination is involved in terminal differentiation, apoptosis, necrosis, NET formation and developmental processes[295]. Since PAD4 and PAD2 proteins have been deeply investigated for their involvement in distinct disease pathogenesis, many studies have focused their attention mainly on these two isoforms[304,305]. During terminal differentiation of keratinocytes, keratin is citrullinated by PAD1 altering its structure, thereby promoting binding to other proteins. Filaggrin is also deiminated, facilitating its cleavage by proteases. Filaggrin then bundles with keratin into a 3D structure that creates a matrix much more resistant to insults[306]. In the CNS, PAD2 is expressed mainly by oligodendrocytes, astrocytes and microglial cells. Citrullination of MBP is probably essential for the plasticity of the CNS in young age as the ratio of citrullinated MBP and total MBP changes rapidly after postnatal life. PADs are also involved in gene regulation. Inregards, PAD4 has been shown to target arginine and methylarginine of H3 and H4, thus, disabling histone methylation on these amino acids. For example, PAD4 has been shown to interact with regulatory elements of p53, such as p53-binding sites on the p21 promoter, where it citrullinated histones preventing p53 binding and subsequent gene repression[307]. Several studies have confirmed a reciprocal action of PAD4 and p53. Tanikawa et al. have shown that p53 transactivated PAD4 through

an intronic p53-binding site[308]. Due to DNA damage, several proteins became citrullinated, but the silencing of PAD4 or p53 significantly inhibited their citrullination. This indicates that protein citrullination is regulated in a p53/PAD4-dependent manner. Estrogens also seem to regulate PAD4: Dong et al. have reported that PAD4 expression is activated by 17 β-estradiol: on one hand, the complex of estrogen receptor (ER) and estrogen binds to the estrogen responsive element of PAD4 promoter to induce its expression; on the other hand, estrogen-ER complex enhances AP-1 binding to the PAD4 promoter increasing its expression[309]. PADs are also involved in immune regulation. The cells of the hematopoietic lineage (especially, monocytes and granulocytes) express PAD4, suggesting that citrullination has a key role in the physiological function of these cells. Regarding immune functions, one of the most recently discovered neutrophil defense mechanisms is the trapping and killing of bacteria by forming highly decondensed chromatin structures, termed neutrophil extracellular traps (NETs). Neutrophils isolated from PAD4-knockout mice lost their ability to release NET and histone hypercitrullination was not detectable, with PAD4-knockout mice being more susceptible to bacterial infections[310]. It has been shown that after translocation of PAD4 from the cytoplasm to the nucleus, hypercitrullination of histones leads to decondensation of chromatin and subsequent expansion, until the membranes break out releasing a sticky net of DNA mixed with granular antimicrobial factors, the so-called NETS, that trap and kill bacteria and other pathogens[311]. Citrullination of chemokines has been also demonstrated and seems to reduce the potency of their activity. Citrullinated CXCL8 (IL-8) has reduced affinity to glycosaminoglycans (heparin), is resistant to thrombin- or plasmin-dependent cleavage into a more potent CXCL8 fragment, and is unable to attract neutrophils to the peritoneum. Cytokines also influence PAD activity: TNF treatment induces the translocation of PAD4 from the cytosol to the nucleus in oligodendroglial cell lines[312]. Transgenic mice overexpressing TNF have increased levels of citrullinated histones and elevated nuclear PAD4. These examples disclose a complex function of PADs with mainly anti-inflammatory and antibacterial effects[313].

2.2 The role of citrullination in the pathogenesis of AID, cancer, neurodegenerative diseases

PADs have a role in maintaining many vital cellular processes; therefore, it is necessary for a balance in PAD activity or these processes can become uncontrollable with potentially deleterious side effects.

Aberrant/altered citrullination has been observed and associated with several pathologies, firstly with RA, where antibodies recognizing citrullinated proteins are detected in the majority of patients. Later,

it became clear that PADs are involved in the development of other inflammatory conditions, such as AID, neurodegenerative diseases and cancer (Fig.9).

2.2.1 Citrullination and rheumatoid arthritis

Citrullination, a recently discovered form of post-translational modifications, has become the highlight of research due to its potential pathogenetic role in an autoimmune condition, RA. Antibodies produced against citrullinated proteins are referred to as ACPAs, which are major diagnostic and prognostic factors of RA, and also influence the age at disease onset[314].

The term "rheumatoid arthritis" RA was defined in 1859 by Alfred Baring Garrod to distinguish the disease from other forms of arthritis (osteoarthritis, spondyloarthritis etc.). It is a T-cell-driven AID, accompanied by autoantibody production triggering local and systemic manifestations. The main symptoms are prolonged pain and stiffness of the joints. RA affects 0.5-1 % of the worldwide population with higher prevalence in developed countries. RA affects patients between 20 and 40 years and is more common in women, probably due to hormonal effects. The chronic inflammatory response is at first affecting the connective tissue of the joint, known as the synovium, terminally resulting in destruction of articular structures. In some cases, extra-articular manifestations such as vasculitis can even end up fatal[315,316].

The major treatments include non-steroidal anti-inflammatory drugs (NSAIDs), steroids, disease-modifying antirheumatic drugs (DMARDs) and biologics.

At the immunological level, great effort has been made to unveil the complex etiopathogenesis of RA. It has been shown that the inflammation is mediated by immune complexes (IC) composed of autoantibodies bound to their cognate autoantigens. Under physiological conditions, IC fixing complement are removed by professional phagocytes from the circulation[317]. In the context of autoimmunity, the persistent production of autoantibodies leads to permanent formation and deposition of IC at the site of inflammation (in the case of RA in the joints) causing chronic inflammation and subsequent tissue damage. After fixation of complement, neutrophils and monocytes are attracted by anaphylatoxins such as C3a and C5a, products of complement cleavage, to the site of IC deposition. There they get activated, engulf IC and release proteolytic enzymes. This vicious cycle fueled by autoantibody production and IC deposition slowly macerates the synovial tissue[315]. Autoantibodies isolated from patients with RA were shown to recognize citrullinated proteins (anti-citrullinated peptide antibodies, ACPA) and IgG (rheumatoid factor, RF)[318]. The observations that RF is a hallmark of many patients affected by RA and that it binds to the Fc part of autologous IgG, suggests that the pathology of RA might be the effect of an autoimmune reaction mediated by autoantibodies[319]. RF was the first marker used for the diagnosis of RA. Recently, ACPA

a more promising diagnostic marker due to its higher specificity (98 % instead of 70 % in case of RF) is used in the clinic[320]. Accordingly, patients with RA are divided into two subsets regarding their ACPA serostatus[321]. These autoantibodies are found in a subset of patients with RA already years before the onset of the disease and predict a more aggressive and severe progression. Thus, a therapeutic approach targeting the neutralization of ACPA might be highly promising. The correlation of ACPA-positivity with several susceptibility factors, such as HLA-associated predisposition, has led to the development of several models of disease pathogenesis[322–324]. Nowadays much more is known about the etiopathogenesis of ACPA-positive than ACPA-negative patients.

The concept that citrullination is involved in the etiopathogenesis of RA has been supported by several lines of evidence. First, RA is a chronic inflammatory disease and citrullination is an inflammation-associated process. In 2006, Makrigiannakis and coworkers demonstrated that deiminated proteins were present in biopsies of several inflammatory diseases such as RA, inflammatory bowel disease (IBD), and myositis, but not in that of healthy controls, suggesting that this modification is occurring also in other inflammatory settings[325]. Second, the presence of ACPA in the synovial fluid (SF) and serum of patients with RA is an indication for an (abnormal) immune response against citrullinated antigens. Moreover, injection of ACPA into mice aggravated the course of collagen-induced arthritis (CIA) in a dose-dependent manner[326]. Thirdly, in two independent studies PAD2 and PAD4 co-localized with citrullinated proteins in the synovial tissue (ST) of patients with RA[304,327]. Furthermore, the pathogenic role of ACPA is confirmed by its positive correlation with disease activity and severity, as previously described[328]. Surprisingly, infants whose mothers were affected by RA and ACPA-positive did not develop any symptoms characteristic for RA[329]. As a final confirmation for citrullination as a main player in RA, the injection of the pan-PAD inhibitor Cl-amidine (N-α-Benzoyl-N5-(2-Chloro-1-Iminoethyl)-L-Ornithine Amine) into mice with CIA ameliorated the clinical disease score, reduced total citrullination in the synovia and serum, and decreased the levels of IgG2b autoantibodies recognizing collagen and C3 deposits[330]. However, citrullination is not necessarily associated with immunogenicity in RA. In 2006, Matsuo et al. showed that citrullination primed the immunogenicity of one antigen, however, antibodies recognizing the uncitrullinated counterpart have also been detected in a cohort of patients with RA[331].

The finding that aberrant citrullination is not only correlated with RA, but has been found in patients with other chronic inflammatory conditions, indicates that **citrullination is an inflammation-dependent process** rather than RA-specific[325].

This finding is supported by the fact that citrullination has later been observed and associated with other diseases, such as fibrosis, psoriasis, neurodegenerative diseases, and cancer (Fig.9)[332].

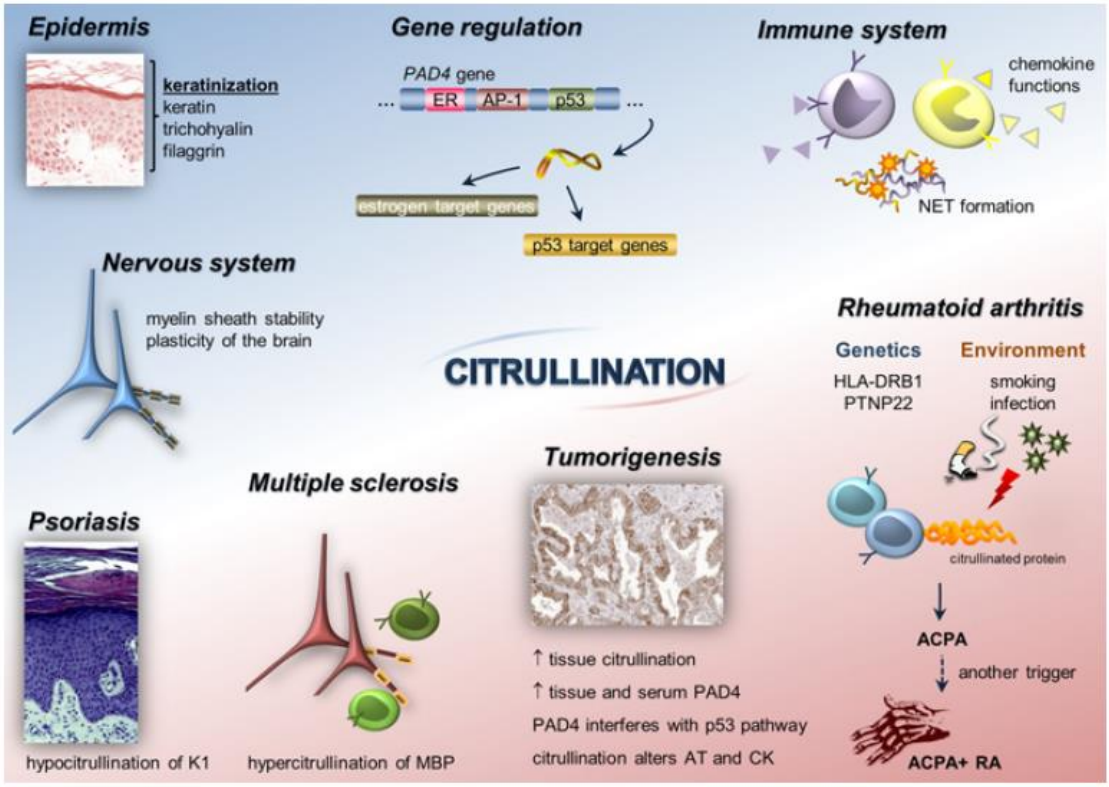

Figure 9

The role of citrullination in physiological and pathological conditions[313].

2.2.2 Citrullination and fibrosis

The connection between citrullinations and pathology is for example found in pulmonary and liver fibrosis. In both cases, the expression of PAD2 and PAD4 mRNA is elevated. In idiopathic pulmonary fibrosis (IPF), there is an increased level of citrullinated proteins in bronchoalveolar lavage fluid (BALF) compared to controls[333]. Since smoking is associated with an increased level of citrulline in BALF as well, this may explain why smokers are more susceptible for RA[334]. Recently, it was demonstrated that increased levels of citrullinated VICM (citrullinated and MMP-degraded vimentin) was present in a CCl4 rat model of liver fibrosis and in patients with hepatitis and non-alcoholic fatty liver disease with early fibrosis[335]. In another study, it was observed that serum concentrations of anti-modified citrullinated vimentin (anti-MCV) were able to separate patients with moderate/severe fibrosis or cirrhosis from patients with no fibrosis[336].

2.2.3 Citrullination and psoriasis

The word psoriasis is derived from the Greek word psora, meaning ‘‘itch’’. Psoriasis is a chronic skin disease characterized by inflamed lesions covered with silvery-white scabs of dead skin. In psoriasis, keratinocytes proliferate very rapidly and travel from the basal layer to the surface in only about four

days. This process normally takes about a month. The skin cannot shed these cells quickly enough so they accumulate in thick, dry patches, or plaques. In normal keratinocytes, keratin K1 is citrullinated by PAD1 during terminal differentiation. This process causes the keratin filaments to become more compact, which is essential for the normal cornification process of the epidermis. The keratinocytes in the psoriatic hyperproliferative plaques do not contain citrullinated keratin K1[337]. In this ‘‘chicken and egg’’ situation it is not clear whether the increased cell proliferation prevents adequate citrullination by PAD1 or that inactivity of PAD1 allows hyperproliferation and accumulation of keratinocytes. Whether the absence of citrullination is associated with a defective PAD1, is unknown. Thus, although the mechanism is unknown, aberrant citrullination in psoriatic epidermis obviously is related to PAD1.

2.2.4 Citrullination and neurodegenerative diseases

MS is a chronic inflammatory disorder of the CNS, characterized by destruction of the myelin sheath. It is the leading cause of neurological deficit in North America and Western Europe[338].
Pathologic findings include multiple sharply demarcated areas of demyelination throughout the white matter of the CNS. The myelin sheath is formed by a single layer of cells that cover the axons of neurons. These cells, oligodendrocytes in the CNS and Schwann cells in the peripheral nervous system, provide electrical insulation to the neuron, which is important for adequate conduction of nerve impulses. Demyelination causes loss of nerve signals, which in turn results in many different clinical manifestations (e.g., visual loss, extraocular movement disorders, paresthesias, loss of sensation, weakness, dysarthria, spasticity, ataxia, and bladder dysfunction). The cells of the myelin sheath form a multibilayer structure around the axons consisting of lipid–protein complexes in a ratio of about 3:1. Two major proteins, MBP and lipophilin, account for 85% of the protein fraction. MBP is a highly cationic protein, capable of forming strong interactions with negatively charged phospholipids such as phosphatidylserine[339]. In approximately 18% of the MBP molecules of healthy adult humans, 6 (out of 19) arginines are citrullinated (MBP-cit6: R25, R31, R122, R130, R159, R169)[340]. The remaining MBP molecules do not contain citrulline. In MS patients, the proportion of MBP-cit6 is increased to 45% of total MBP[341]. The decreased net positive charge of MBP-cit6 causes partial unfolding of MBP molecules and weakens their interaction with the phospholipids. Although MBP-cit6 is capable of forming lipid complexes more rapidly than non-citrullinated MBP, the complexes that are formed are not as densely packed as those formed with non-citrullinated MBP[342]. In children up to the age of 2 years, almost all MBP is of the MBP-cit6 type. The ability to rapidly form myelin structures is important in young children with a developing nervous system, while good electric insulation of the axons is less important, because of the smaller distances that have to be

bridged. The amount of MBP-cit6 drops rapidly from almost 100% to 18% between 2 and 4 years of age[343]. Interestingly, this is the infant phase associated with the greatest motoneuronal development. Citrullination of MBP not only alters the lipid-complex forming ability of MBP, it also unfolds the structure of MBP, making the protein more susceptible to degradation by cathepsin D. MBP-cit6 is degraded four times more rapidly by cathepsin D than non-citrullinated MBP. In a rare case of acute fulminating MS (Marburg type), 80% of the MBP molecules are heavily citrullinated (MBP-cit18)[344]. This highly citrullinated form of MBP is not capable of forming lipid complexes. The severely unfolded MBP-cit18 is degraded 45 times more rapidly by cathepsin D than normal MBP[293].

Transgenic mice bearing high copy numbers of a transgene coding for DM-20 (a myelin proteolipid protein) spontaneously develop a pathology with much similarity to MS[345]. The amount of citrullinated MBP in these mice is increased 2- to 3-fold compared to control animals[346]. The onset of symptoms is preceded by an increase in the amount of myelin-associated PAD2 protein. Of interest, phosphatidylserine, one of the major lipid components of myelin enhances the velocity of the reaction catalyzed by PAD2 and reduces the Ca^{2+}. Currently, clinical trials with paclitaxel, the active component of the anti-cancer drug taxol, showed that can inhibit citrullination of MBP by PAD2 in vitro[347]. Treatment with paclitaxel attenuates clinical symptoms and induces remyelination of damaged sheaths, underlining the possible importance of PAD as a candidate factor in demyelinating disease[348].

Citrullination has been correlated with various other neurodegenerative diseases, such as Alzheimer's (AD) and Parkinson's (PD) disease.

AD is the most common form of dementia, constituting 60–80% of all dementias. A person showing symptoms of AD can have difficulty in remembering words, suffer from depression and show behavioural changes and confusion. The benchmark of AD confirmation is the presence of two key neurohistological hallmarks namely beta amyloid (Aβ) plaques, and neurofibrillary tangles (NFTs) comprising hyper-phosphorylated tau protein in specific anatomical regions of the brain. The relevance of humoral responses in the pathophysiology of AD, is little understood; however, it has been hypothesized that autoantibodies against citrullinated nerve tract covering myelin sheath proteins are implicated in the development of AD. Damaged myelin interacting with Aβ deposits in AD and antibodies to glial derived antigens are reported[349]. Acharya et al. showed that autoantibodies in AD associate with protein citrullination and PAD4, which targets pyramidal neuronal intracellular proteins in the AD hippocampus[350]. Matsuomi et al. found an abnormal accumulation of citrullinated proteins and an increase of the PAD2 content in the hippocampi of AD patients[351]. The most over-citrullinated proteins in AD were structural proteins such as vimentin, MBP and glial fibrillary acidic protein (GFAP). Overexpression of PADs and protein citrullination are abnormal features of

neurodegeneration and inflammatory diseases and have actually been proposed as a possible cause of AD[352,353]. PAD2 and PAD4 enzymes are mainly detected in astrocytes and neurons, and there is a concomitant accumulation of citrullinated proteins within PAD4-expressing cells including neurons of the hippocampus and the cerebral cortex[350,351]. This implies that citrullination of neuronal cytoskeletal proteins may be toxic, because disease-associated neuronal loss appears to result in the release of their cellular contents into the cerebral parenchyma from which they enter the blood and lymph circulation. Some of them are able to elicit an immune response that results in the production of autoantibodies[350]. Inhibition of PAD may therefore be worth serious therapeutic consideration in the treatment/prophylaxis of diseases where citrullination takes place.

Parkinson disease is the second-most common neurodegenerative disorder that affects 2–3% of the population over 65 years of age. Symptoms include a disorder of movement, palsy, cognitive impairment, autonomic dysfunction, disorders of sleep, depression and hyposmia (impaired smell).

Neuronal loss in the substantia nigra, which causes striatal dopamine deficiency, and intracellular inclusions containing aggregates of α-synuclein are the neuropathological hallmarks of Parkinson disease[354].

In PD citrullinated proteins were shown to be mislocalized: in patients with PD they were found within the cytoplasm of many surviving dopamine neurons in the human substantia nigra (SN), while in control sections citrullinated proteins appeared as small fibers surrounding dopamine neurons that co-localized with GFAP[353]. *In vitro* studies demonstrated that amyloid peptides bind to PAD2, resulting in catalytic fibrillogenesis and formation of insoluble fibril aggregates[355].

To conclude, PAD has shown to play an important role in the onset and progression of neurodegenerative diseases and that citrullinated proteins/PAD may become a useful marker for their diagnosis[356].

2.2.5 Citrullination and cancer

There is a body of evidence that PAD4, but also PAD2 and PAD1, and citrullination play a role in tumorigenesis with promising implication for targeted cancer therapy. Measuring the corresponding concentration of PAD enzymes appears to be a robust way of estimating the frequency of this posttranslational modification. Inregards, PAD4 expression levels were found increased in 12 different tumors relative to their respective normal counterparts, including breast carcinomas, lung adenocarcinomas, hepatocellular carcinomas, esophageal squamous cancer cells, colorectal adenocarcinomas, renal cancer cells, ovarian adenocarcinomas, endometrial carcinomas, uterine adenocarcinomas, bladder carcinomas, chondromas, as well as other metastatic carcinomas[357]. In addition, Chang and Han have found high tissue expression of PAD4 and citrullination in various

malignant tumors, but not in benign tumors or non-tumourous tissues, whereas metastasis exhibited much higher PAD4 levels compared with corresponding primary tumors[358], implicating citrullination involvement in the progression from benign neoplasm to invasive malignancy.

The mechanisms by which PAD4 interferes to promote tumoral transformation are diverse. Most likely, it interferes with regulation of gene expression. First of all, it is known that PAD4 can translocate to the nucleus to citrullinate histones, with H3 and H4 as main targets, and transcription factors[296,359]. As already mentioned, multiple interactions of PAD4 and p53 were reported, suggesting the importance of PAD-induced citrullination in apoptosis[308]. Expression of a major p53 target gene OKL38 was repressed by a p53-mediated recruitment of PAD4 to the promoter of OKL38 and subsequent removal of histone arginine methylation mark, thus directly modulating apoptosis[360]. Interaction of PAD4 with p53 was shown to citrullinate H4R3 upon chemotherapy treatment, with citrullinated regions being colocalized with decondensed soluble chromatin in apoptotic cells, indicating the direct involvement of the PAD4– pp53 complex in apoptosis. PAD4-/- mice displayed apoptosis resistance, whereas patients with lung cancer with citrullinated H4R3 expression had smaller tumor size.

PAD4 was found to bind and subsequently citrullinate the inhibitor of growth 4 (ING4), another tumor suppressor protein known to interact with p53[361]. PAD4-driven citrullination of ING4 at the nuclear localization sequence region prevented p53-to-ING4 binding, repressed p53 acetylation, and subsequently inhibited downstream p21 expression. In line with this, a PAD4 inhibitor has been shown to display cytotoxic effects on various cancerous cell lines[362].

PAD4 was also reported to play a role in Epithelial-to-Mesenchymal transition (EMT), thereby promoting cancer progression. To this end, knockdown of PAD4 in breast cancer cells activated TGFβ signaling via upregulation of Smad4, and induced EMT by reducing E-cadherin and promoting vimentin expression, further propelling cell invasiveness[363]. These effects were dependent on PAD4-driven citrullination of transcription factor glycogen synthase kinase 3 β (GSK3β), which resulted in a translocation of GSK3β from the cytoplasm into the nucleus and initiation of multiple gene expression involved in tumor trasformation[363].

PAD2 has also been associated with tumorigenesis through H3-mediated citrullination, that correlated with increased expression of human epidermal growth factor receptor 2 (HER2) and other 200 genes induced upon activation of the ER. Moreover, it was found that PAD2 levels are increased in breast cancer cell lines[364].

On contrary, a downregulation of intertumoral PAD2 expression was identified in a cohort of patient with colorectal cancer specimens in comparison with normal mucosa of healthy control tissues[365]. *In vitro* overexpression of *PADI2* was shown to suppress proliferation of colon cancer cells in

association with increased protein citrullination. The growth defect induced by *PADI2* was accompanied by arrest of cell cycle progression in G_1 phase. Fukuyama et al. concluded that *PADI2* suppresses the proliferation of colonic epithelial cells through catalysis of protein citrullination, and that downregulation of *PADI2* expression might therefore contribute to colon carcinogenesis.

Regarding PAD1, only one study showed PAD1 expression upregulated in patients with triple-negative breast cancer (TNBC) based on bioinformatics studies. The TNBC refers to the breast cancer tested negative for ER, progesterone receptor (PR) and HER2, and it is the most invasive and aggressive among the breast cancer subtypes. One of the first steps of metastasis is the degradation of the basement membrane and stromal extracellular matrix (ECM), which form physical barriers to restrict cell movement. During this process, matrix metalloproteinases (MMPs) are involved in the breakdown of the ECM components, thereby facilitating tumor progression and metastasis. Knockdown or silencing of PAD1 reduced MDAMB- 231 cancer cell proliferation, migration, and invasion *in vitro*. Further, silencing of PAD1 reduced MMP expression and reversed the EMT *in vitro* through direct citrullination of MEK1, that disrupted MEK1-mediated phosphorylation of ERK1/2, leading to MMP2 overexpression[366].

Nowadays multiple research groups provided solid evidence of citrullination being in control of several steps of cancer progression, such as invasiveness, metastasis, angiogenesis. In addition, several irreversible inhibitors for these enzymes, including Cl-amidine, have proved their efficacy in multiple *in vitro* and *in vivo* experiments[358,367,368]. Importantly, pharmacologic inhibition of PADs substantially reduced proliferation of cancer cells while not affecting the viability of normal cells, potentially opening avenues for targeted therapy[369].

Taken together, these data justify the feasibility of testing PAD inhibitors in preclinical models of cancer.

In Fig.10 pathways affected by PAD1, PAD2 and PAD4-mediated citrullination in order to promote tumorigenesis.

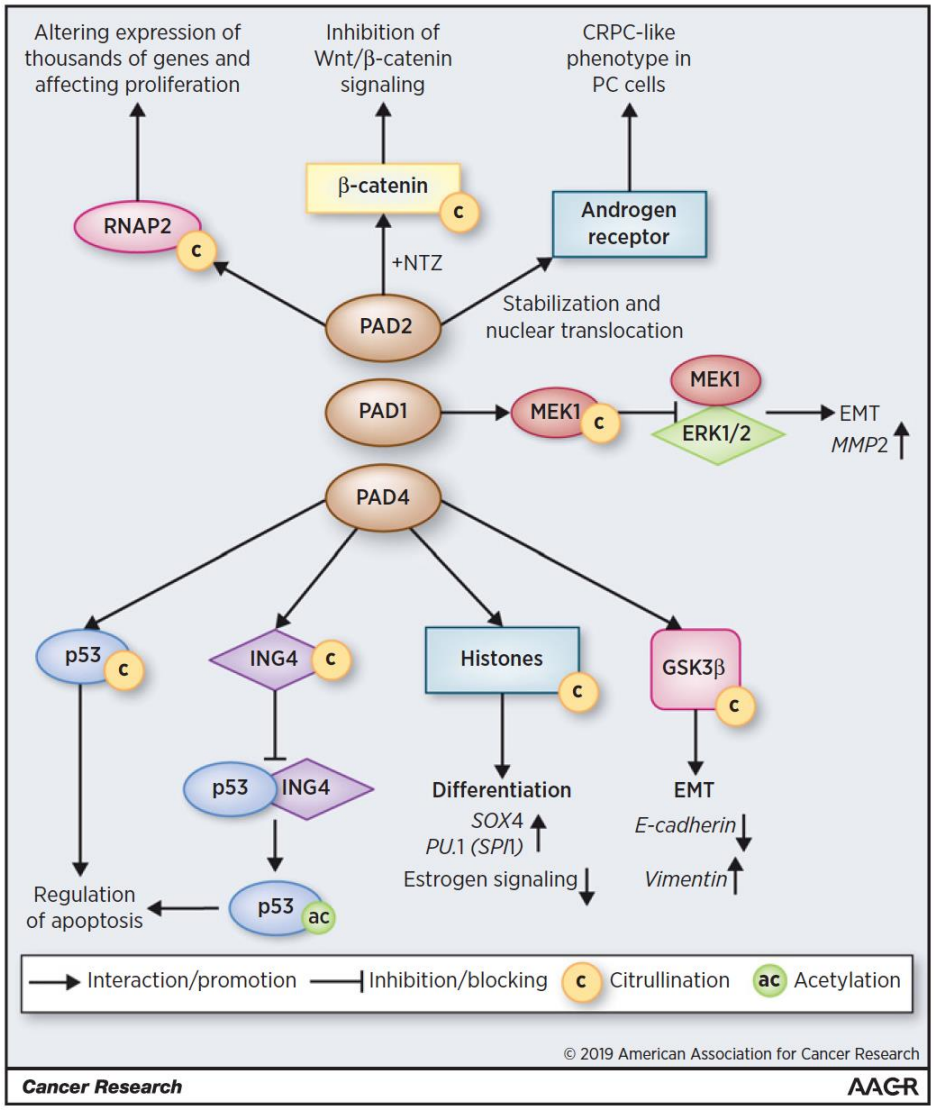

Figure 10

Schematic illustrating how PAD-mediated citrullination may affect cell signaling to facilitate cancer progression. PC, prostate cancer[369].

2.3 Citrullination and viral infections

The high specificity for RA and the development of citrullinated substrates that allow easy and reliable detection of these autoantibodies have boosted clinical interest in ACPAs as a new diagnostic tool. Besides this diagnostic application, the presence of ACPAs is associated with more severe joint destruction and greater disease activity, with ACPA positivity at the time of diagnosis being an important predictor of a more aggressive disease course[370,371].

Recent studies showed the presence of ACPA also in several infectious diseases, such as tubercolosis, leishmaniosis, Hansen's disease, atypical mycobacteriosis, hepatitis B and C virus, HIV, Human T-lymphotropic virus (HTLV-1), mononucleosis, Chagas disease, Yersinia[372]. However, there are only

few studies that evaluated the role of viral infections in the development of AIDs, and their association with citrullination.

Interestingly, in 2006 Pratesi hypothesized that citrullination of a viral protein may create epitopes that are recognized by anti-filaggrin antibodies (AFAs), which bind citrullinated filaggrin, a protein involved in the aggregation of cytokeratin filaments, and synthesized in epithelial cells[373]. During cell differentiation, the precursor profilaggrin is dephosphorylated and cleaved; at this stage, the arginyl residues of filaggrin are converted into neutral citrullyl residues by PAD, generating more acidic isoforms[374].

A comparative evaluation of the sequences recognized by AFAs showed that their most crucial feature is the presence of citrulline flanked by neutral amino acids such as glycine, serine, or threonine[375]. Similar amino acid repeats are often found in nucleic acid– binding proteins; some of these are of viral origin (e.g., the transcription-regulating proteins in herpesvirus. EBV nuclear antigen 1 (EBNA-1), one of few encoded EBV proteins, contains in its N-terminal region a sequence (amino acids 35–58) characterized by a 6-fold Gly–Arg repeat[376].

Eventually, antibodies isolated from 45% of RA patients recognize specifically the peptide corresponding to the EBNA-1 135–58 sequence containing citrulline in place of arginine, indicating that ACPAs react with a viral deiminated protein and suggest that EBV infection may play a role in the induction of these RA-specific antibodies[377].

Moreover, anti-viral citrullinated protein (VCP) antibodies immunoprecipitated an 80-kd band from the lysate of calcium ionophore–stimulated lymphoblastoid cell lines, that was reactive with a monoclonal anti–EBNA-1 antibody and with anti–modified citrulline antibodies. In a later study by Pratesi et al., viral citrullinated peptide 2 (VCP2), a peptide corresponding to the modified 338–358 sequence of the EBNA-2 protein, was synthesized by substituting each arginine with citrulline, and then analysed its potential as a substrate for the detection of ACPA. Anti-VPC antibodies have been found in approximately 66% of RA patients sera but only in 5% of controls. This parameter also correlated positively with anti-CCP level, indicating that VCP could represent a valid substrate for ACPA detection. In addition, they showed that affinity-purified anti-VCP2 antibodies immunoprecipitated deiminated Epstein–Barr virus nuclear antigen (EBNA-2) from an EBNA-2-transfected cell line, suggesting that viral sequences may be involved in the generation of the ACPA response.

Recently, a study conducted in Denmark aimed to analyze antibody reactivity to citrullinated EBV nuclear antigen-2 (EBNA-2) peptides from three different EBV strains (B95-8, GD1 and AG876) using streptavidin capture enzyme-linked immunosorbent assay[378]. One peptide, only found in a single strain (AG876), obtained a sensitivity and specificity of 77% and 95%, respectively and

showed high sequence similarity to the filaggrin peptide originally used for ACPA detection. Interestingly, the single peptide is recognized specifically by RA sera and is able to compete with assays containing multiple peptides, making an interesting candidate as substrate for RA detection. Nevertheless, these finding promote shed light on a potential role of EBV as environmental factor contributing to the onset and/or development of RA. According to that, a recent paper by 'tHart et al. proposed the hypothesis that EBV infection could induce MS progression by inducing citrullination of peptides in conjunction with autophagy during antigen processing, endowing B cells with the capacity to cross present autoantigen to aggressive CD8+CD56+ T cells, thereby leading to MS progression[379].

2.4 PAD inhibitors

Since the importance of citrullination in the pathogenesis of RA and other associated pathologies has become evident, the number of studies on the development of PAD inhibitors has increased.

The first PAD inhibitor was developed in 2006 by Paul R. Thompson and colleagues, who described the synthesis and characterization of F-amidine (Fig.11)[380]. The initial design of F-amidine was based in part on its structural homology to N-α-benzoyl Arg amide (BAA), one of the best small molecule PAD4 substrates [kcat/Km = 1.1 Å~ 104 M−1 s−1 (14), and can be considered to consist of two major moieties, a fluoroacetamidine-based warhead and a specificity determinant that was expected to target the warhead to the active site of PAD4, where it will react with C645 to form a stable thioether adduct via one of two potential mechanisms[381].

Subsequent studies showed that the warhead itself can act as a selectivity determinant.

Cl-amidine was one of the first more potent PAD inhibitor, with a *k*inact/*K*I of 13000 M-1s-1 for PAD4 (compared to F-amidine *k*inact/*K*I di 3000 M-1s-1), that irreversibly inhibits all of the known active PAD isozymes (i.e., PADs 1–4) with low micromolar potency; the ability of Cl-amidine to inhibit PAD6 has not been assessed because *in vitro* activity has not been detected for this isozyme (Fig.11). The structure of Cl-amidine is similar to BAA, except that it incorporates a reactive haloacetamidine warhead in place of the substrate guanidinium. In addition to the PAD inhibition *in vitro*, Cl-amidine has been also evaluated in a cell-based assay of PAD4 activity. This is a mammalian two-hybrid assay that monitors the enhanced interaction between the glucocorticoid receptor-interacting protein 1 binding domain of p300 and glucocorticoid receptor-interacting protein 1 on intracellular PAD4. This assay demonstrated that Cl-amidine is cell permeable, can transit membranes, and inhibit PAD4 activity in the nucleus[380].

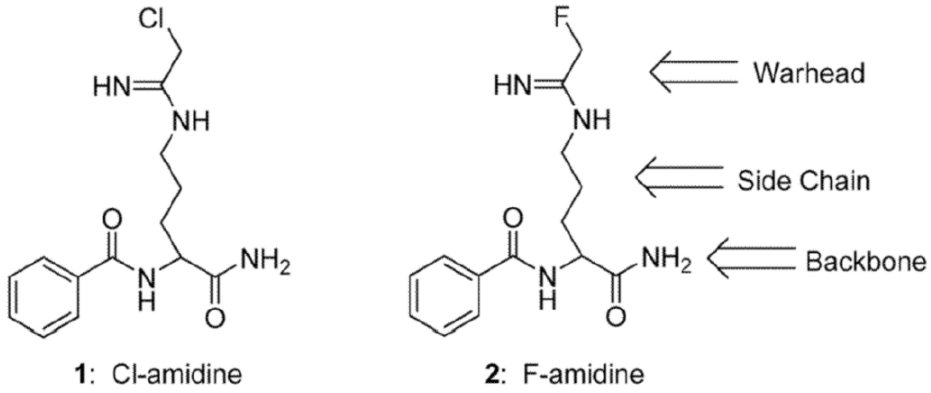

Figure 11

Structure of Cl-amidine (left) and F-amidine (right)[330].

Figure 12

Structure of N-[(1S)-1-(1H-benzimidazol-2-yl)-4-[(2-chloro-1-iminoethyl)amino]butyl]-[1,1'-biphenyl]-4-carboxamide (BB-Cl-amidine).
BB-Cl-amidine is a C-terminal bioisostere of Cl-amidine. The Cl-amidine structure is based on the structure of benzoyl arginine amide, a small molecule PAD substrate. In BB-Cl-amidine, the C-terminus is replaced by a benzimidazole to prevent proteolysis of the C-terminal amide, and the N-terminal benzoyl group is replaced by a biphenyl moiety to increase hydrophobicity and enhance cellular uptake[382].

Notably, first-generation pan-PAD inhibitor Cl-amidine demonstrates efficacy in animal models of RA, lupus, ulcerative colitis, spinal cord injury, breast cancer, and atherosclerosis[330,382–386]. However, in an effort to design compounds with increased potency *in cellulo*, metabolic stability and cell membrane permeability, the C-terminal carboxamide was replaced with a benzimidazole moiety to yield BB-Cl-amidine (Fig.12). The N-terminal benzoyl group is replaced with a biphenyl moiety, which was incorporated to increase hydrophobicity and thereby facilitate cellular uptake[368,382].
BB-Cl-amidine and Cl-amidine have similar potency and selectivity, but *in cellulo* BB-Cl-amidine is 20-fold more potent, probably due to its greater bioavailability and increased uptake. In addition, BB-Cl-amidine showed a significantly longer *in vivo* half-life than Cl-amidine.

In vivo studies showed that BB-Cl-amidine reduces the formation of NETs and protects from vascular damage in lupus-prone New Zealand murine models, such as MRL/*lpr*[382].

Inactivation by amidines occurs via the initial attack of the Cys645 thiolate on the amidinium carbon, which results in the formation of a stable protonated tetrahedral intermediate that mimics the initial tetrahedral intermediate formed during substrate hydrolysis; His471 is the likely proton donor. The intramolecular halide displacement reaction then proceeds to generate a three-membered sulfonium ring. Deprotonation and collapse of the tetrahedral intermediate leads to a 1,2-shift that generates a thioether adduct, the existence of which has been verified crystallographically (Figure 13)[387].

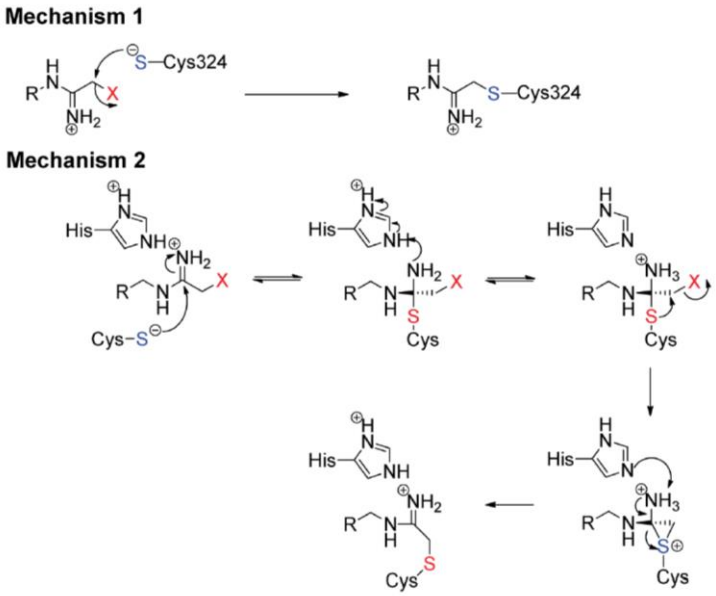

Figure 13

The proposed mechanisms of PAD inactivation by halo-acetamidine based inhibitors[387].

Since the different PAD isoforms have distinct tissue expression in physiological and pathological conditions, in order to study their biological role and develop a targeted therapy specific PAD inhibitors are necessary.

Although the association between dysregulated PAD activity and human disease seems clear, especially with the recent demonstration that Cl-amidine decreases disease severity in the CIA model of RA and the dextran sodium sulphate (DSS) model of Ulcerative Colitis, what is not known are the specific identities of the isozymes involved in the onset and progression of these pathologies. From the available evidence, the most likely candidates appear to be PAD2 and 4, as these enzymes are expressed by immune cells and both are overexpressed in these several pathological conditions[388].

For this reason, Thompson and colleagues mainly focused on the development of a PAD2-selective inhibitor named 30a, where Protein arginine deiminase 2 (PAD2) plays a key role in the onset and

progression of multiple sclerosis, rheumatoid arthritis and breast cancer. To achieve this goal, they synthesized a series of benzimidazole-based derivatives of Cl-amidine and demonstrated that substitutions at both the N-terminus and C-terminus of Cl-amidine result in >100-fold increases in PAD2 potency and selectivity (30a, 41a, and 49a) as well as cellular efficacy 30a[389].

An interesting PAD4 inhibitor has been already developed by Lewis and colleagues, named GSK199. This was the first reversible selective PAD4 inhibitor. GSK199 takes advantage of the residue Phe634, which is not conserved in other human PADs. GSK199 demonstrates high affinity binding to the low-calcium form of PAD4, probably explaining the reversible inhibition process[390].

In addition, threonine-aspartic acid-F-amidine (TDFA) provided PAD4-selective inhibition[391], D-Cl-amidine provided PAD1-selective inhibition[392], while Cl4-amidine and a series of hydantoin- based inhibitors provided PAD3-selective inhibition[393,394]. While these inhibitors have brought about significant advances in PAD inhibitor development, most of them exhibit poor efficacy in cell-based assays (potentially due to metabolic instability and poor membrane permeability) that better clarify the incessant research after new inhibitors.

The clinical application of these inhibitors is not approved, yet, however, it might be a highly promising and specific approach.

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MANUSCRIPT IN PREPARATION & PUBLICATIONS

SUBMITTED MANUSCRIPT

Human cytomegalovirus-induced host protein citrullination is essential for viral replication

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Post-translational modifications (PTMs) are a key virulence strategy exploited by pathogens to subvert the host innate immune response. Citrullination, the conversion of arginine to the non-genetically encoded amino acid citrulline, is a PTM catalyzed by protein arginine deiminases (PADs). Although PAD dysregulation has been implicated in a number of inflammation-dependent processes, such as AIDs, neurodegenerative disorders and cancer, there is to date no evidence linking citrullination to viral infection. Human cytomegalovirus (HCMV), a paradigm for viral immune evasion, manipulates host protein functions both transcriptionally and post-translationally to gain a survival advantage. Here, we show that HCMV infection triggers citrullination of host proteins in primary human fibroblasts and that pharmacological or genetic inhibition of specific PAD isoforms strongly impairs HCMV replication, indicating that PAD-mediated citrullination is required for HCMV pathogenicity. Furthermore, by citrullinome analysis we show that several interferon (IFN)-inducible cellular host but not viral proteins are citrullinated following HCMV infection. Consistently, their siRNA-mediated knockdown increases the production of infectious HCMV virions, suggesting that citrullination constitutes a novel mechanism of HCMV immune evasion. Overall, the key role played by PADs in HCMV-induced citrullination makes these enzymes and their substrates attractive druggable targets for the treatment of HCMV-related diseases.

Human cytomegalovirus (HCMV) is a β-herpesvirus infecting 40-90% of the adult human population. Even though HCMV infection is frequently harmless in healthy patients, it can lead to serious health consequences in individuals with a deficient immune system, such as transplant recipients and AIDS patients[1]. In addition, congenital HCMV infection is the most common cause of fetal and neonatal malformations in developed countries[2]. More recently, HCMV has been linked to AIDs and degenerative disorders like arteriosclerosis, vascular disease and immune aging as well as to certain types of tumors[3–8].

One of the strategies devised by HCMV to favor its replication consists in modifying host cellular proteins at the post-translational level, thereby altering their localization, interaction, activation and/or turnover[9]. In an effort to map post-translational modifications (PTMs) during HCMV infection, we have recently come across an unprecedented mechanism of HCMV-induced citrullination of host cellular proteins. Citrullination, also called deimination, is a process where the guanidinium group of an arginine is hydrolyzed to form citrulline, a non-genetically encoded amino acid. This PTM is catalyzed by the calcium-dependent protein arginine deiminase (PAD) family of enzymes, which in humans is composed of five isoforms (PADs 1-4 and 6), with different tissue-specific expression and substrate specificities[10]. Aberrant citrullination is detected in several inflammatory conditions, including rheumatoid arthritis, lupus, Alzheimer's disease (AD), multiple sclerosis (MS), Parkinson's disease, cancer and atherosclerosis, suggesting that it may play a pathogenetic role in inflammation-related diseases[11–18].

To begin to characterize host protein citrullination during HCMV infection, we performed immunoblot analysis of protein lysates obtained from human foreskin fibroblasts (HFFs) infected with HCMV at different time points using the citrulline-specific probe rhodamine phenylglyoxal-based (Rh-PG) fluorophore[19]. We observed an increase in total protein citrullination, especially in the 60 to 100 kDa range, in lysates from HCMV-infected HFFs at 48 and, albeit to a lesser extent, at 96 h post infection (hpi) compared to uninfected control cells (mock) (Fig. 1a).

We next sought to determine whether PADs played a role in enhanced citrullination by

HCMV. RT-qPCR analysis revealed that *PADI2*, *4*, and *6* genes were all expressed at significantly higher levels in HCMV-infected HFFs at 24 hpi compared to mock-infected controls (i.e. ~4-, ~2, and ~1.5-fold, respectively) (Fig. 1b). In particular, both *PADI2* and *4* genes were upregulated at 8 hpi, reaching a peak at 24 hpi and decreasing afterwards (Fig. 1c). Fittingly, PAD2 and 4 protein levels started to increase at 24 hpi and remained elevated for up to 72 hpi (Fig. 1d). By contrast, the other PAD isoforms were expressed at very low (PAD3) or undetectable levels (PAD1 and 6) and were not modulated by HCMV infection (Fig. 1b, and Supplementary Fig. 1a), indicating that PAD2 and 4 are the only PAD enzymes involved in HCMV-induced citrullination.

To gain more insight into the mechanisms responsible for *PADI2* and *4* transcriptional upregulation, we assessed the promoter activity of both *PADI2* and *4* genes taking advantage of a dual luciferase assay system. To this end, HFFs were transiently transfected with luciferase reporter plasmids carrying the wild-type promoter region of either *PADI2* or *PADI4*, or just the empty vector as control. After 24 h of electroporation, cells were infected with wild-type or UV-inactivated HCMV. UV-inactivation of HCMV was confirmed by assessing its ability to produce viral immediate early antigen (IEA) compared to the intact virus (Supplementary Fig. 1b). As shown in Supplementary Fig. 1d, HCMV infection led to robust induction of luciferase activities driven by either the *PADI2* or *PADI4* promoter (~ 7- and 3.5-fold, respectively), whereas UV-HCMV infection failed to induce a similar response, indicating that the synthesis of one or more viral proteins during the initial stage of infection is critical for transcriptional activation of *PADI2* and *4* genes.

We next proceeded to determine which viral gene product mediated upregulation of PAD2, the most potently induced PAD member by HCMV (Figs. 1b and 1c). Treatment of HCMV-infected HFFs with the protein synthesis inhibitor cycloheximide (CHX) completely shut down HCMV-induced PAD2 protein expression at 24 hpi, attesting that *de novo* gene expression is required to upregulate PAD2 (Fig. 1e). Fittingly, UV-inactivated HCMV failed to induce PAD2 (Fig. 1e). In contrast, treatment with the viral DNA synthesis inhibitor phosphonoformic acid (PFA) did not

seem to affect PAD2 upregulation (Fig. 1e), indicating that true late viral proteins are not involved in PAD regulation.

These results together with the observation that the early kinetics of PAD induction (Fig. 1c) paralleled that of viral IE1 or IE2 gene expression raised the hypothesis that IE gene products may play a functional role in PAD induction. Indeed, adenoviral-mediated overexpression of IE1 (AdVIE1) but not IE2 (AdVIE2) led to a substantial upregulation of PAD2 protein expression (Fig. 1f), indicating that IE1 is at least one of the HCMV products regulating PAD gene expression. Consistently, HFFs infected with a recombinant HCMV strain lacking the IE1 protein (AD169ΔIE1) displayed much lower levels of PAD2 protein expression at 48 (hpi) compared to cells transduced with the wild-type control (AD169IE1), an effect that was reversed by AdVIE1 co-infection (Fig. 1g)[20].

Next, we asked whether citrullination induced by HCMV infection was essential for viral replication. To answer this question, we assessed viral plaque formation in HCMV-infected HFFs treated for 1 h prior infection (MOI 1 PFU/mL) with increasing concentrations of Cl-amidine (25-200 μM), a cell-permeable pan-PAD inhibitor. After 7 days of continuous exposure to Cl-amidine, we observed a dose-dependent downregulation of the number of viral particles in HCMV-infected HFFs, with a complete suppression at 100 μM. The IC_{50} of Cl-amidine was found to be 35.79 μM (Fig. 2a). To further corroborate these data, we also measured viral DNA synthesis by RT-qPCR in similarly treated cells. Remarkably, at the low concentration of 25 μM, Cl-amidine treatment reduced the replication rate of HCMV by ~3-fold compared to untreated cells (Fig. 2b). In line with the plaque forming activity data, treatment with 100 μM Cl-amidine completely shut down HCMV replication (Fig. 2b). To rule out the possibility that the antiviral effects were related to the cytotoxic effects of the drug, we assessed cell cytotoxicity by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. To this end, HFFs were treated 1 h before HCMV infection with 100 μM Cl-amidine, infected with increasing MOI (0.01-1) of HCMV and incubated for an additional 144 h in the presence of the inhibitor. At 144 hpi, the number of plaque

forming units (PFUs) was completely suppressed at MOIs ranging from 0.01 to 0.1 (Fig. 2c). Interestingly, Cl-amidine maintained its antiviral effect even at higher HCMV MOIs (0.5-1).

To determine whether the antiviral activity of the compound was limited to HCMV or could be extended to other viruses, we assessed the effect of scalar Cl-amidine concentrations on viral replication of the following viruses: herpesvirus type 1 (HSV-1) and herpesvirus type 2 (HSV-2), two members of the *Herpesviridae* family; a clinical isolate of adenovirus as prototype of non-enveloped DNA virus; and the RNA virus HIV-1_{IIIb}. Interestingly, only HSV-1 and -2 displayed impaired viral growth in Cl-amidine-treated cells (IC_{50}~ 66.2 and 21.2 μM) (Supplementary Fig. 2a), whereas the replication rates of adenovirus and HIV-1_{IIIb} were only marginally affected by the PAD inhibitor (Supplementary Fig. 2b and c, respectively). Of note, the slight reduction in adenovirus replication observed in HFFs cells treated with higher doses of Cl-amidine was probably due to the cytotoxic effect of the compound, as judged by the MTT assay. Thus, altogether these data point to a herpesvirus-specific antiviral activity of Cl-amidine.

To further demonstrate that Cl-amidine reduces HCMV-driven citrullination in HFFs, we assessed total protein citrullination levels using an anti-peptidylcitrulline antibody (clone F95). In line with the results obtained with the Rh-PG probe (Fig. 1a), protein citrullination peaked at 48 hpi and decreased at 96 hpi. As expected, in the presence of Cl-amidine, protein citrullination was efficiently suppressed at either time point (Fig. 2d). Next, PAD enzymatic activity was measured by means of an *in vitro* antibody-based assay using histone 3 as substrate (Fig. 2e). The reliability of the assay was first assessed using increasing amounts of human recombinant PAD2 in the presence or absence of Cl-amidine (Supplementary Fig. 1c). Consistent with the kinetics of PAD2 and 4 protein expression and with the citrullination profile, PAD catalytic activity was enhanced at 24 hpi, peaked at 48 hpi and decreased at 72 hpi. Importantly, HCMV-induced PAD activity was significantly inhibited by Cl-amidine (100 μM) at 48 and 72 hpi (Fig. 2e). Finally, to assess the extent of HCMV replication, total protein extracts from HCMV-infected HFFs treated with or without Cl-amidine at various pi time points were subjected to immunoblotting using antibodies

against the corresponding IEA, early (ICP36) and late (pp28) proteins. While Cl-amidine treatment only marginally affected IEA expression, it inhibited the expression of viral early and late genes (Fig. 2f), indicating that PAD enzymes support the HCMV productive cycle by fostering the expression of early and late genes.

To confirm that the antiviral activity of Cl-amidine was due to PAD inhibition and not to an off-target effect of the compound, we generated PAD knockout (KO) HFFs using clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 technology. Primary cell lines carrying mutations in genes encoding PAD1 (PAD1 KO), PAD2 (PAD2 KO), PAD3 (PAD3 KO), PAD6 (PAD6 KO), PAD4 (PAD4 KO) or both PAD2 and PAD4 (PAD2/4 DKO) were generated using five different guide RNAs (gRNAs). Tracking of indels by decomposition (TIDE) analysis showed an overall knockdown efficiency ranging from 35% to 45% for each of the PAD KO cell lines (Supplementary Fig. 3). Consistently, immunoblot analysis revealed a reduction in PAD2, 4 and 3 protein expression levels of approximately 56%, 50% (Fig. 3a) and 60% (Supplementary Fig. 4a), respectively. With regard to PAD1 and 6, we could only rely on the aforementioned TIDE analysis (Supplementary Fig. 3) as the expression levels of these two PAD isoforms were barely detectable in HFFs, even after HCMV infection (Supplementary Fig. 1a). Although we failed to achieve total suppression of PAD gene expression, the overall knockdown efficiency proved to be sufficient enough to allow us to perform subsequent functional experiments.

To assess whether PADs are required for HCMV replication, we performed standard plaque assay and quantitative real time RT-PCR as described above. As shown in Fig. 3b, PAD2 and PAD4 KO cells displayed robust inhibition of HCMV replication at MOI 0.1. Interestingly, the double KO cell line for PAD2/4 DKO showed a stronger suppression of viral replication compared to single KO cells, suggesting that the combined action of PAD2 and 4 deiminases potentiates viral replication (Fig. 3b). In line with these results, a consistent reduction of ICP36 protein level was also detected in infected PAD2, PAD4 KO and PAD2/4 DKO cell lines at 48 hpi (Fig. 3a). As expected, depletion of PAD1 (PAD1 KO), 3 (PAD3 KO) or 6 (PAD6 KO) did not affect HCMV

replication rates compared to control (Supplementary Fig. 4b). Thus, PAD2 and 4 but not PAD 1, 3 and 6 sustain the viral cell cycle of HCMV.

To identify which proteins (i.e. cellular and/or viral) were citrullinated during HCMV infection, we performed citrullinome analysis on HCMV infected cells harvested at 48 and 96 hpi. Consistent with our earlier findings, we observed a massive increase in overall protein citrullination in HCMV-infected cells at both 48 and 96 hpi compared to uninfected control cells, even though citrullination levels were slightly diminished at 96 hpi compared to 48 hpi (Fig. 4a and Supplementary Data Set), confirming our previous results (Fig. 1a). Surprisingly, at both time points we failed to detect any citrullinated viral proteins, indicating that HCMV has concurrently evolved unknown mechanisms to circumvent citrullination of its own proteins, which could jeopardize their stability and functionality[21,22]. Using PANTHER software, we identified a wide variety of citrullinated proteins falling into various functional classes, among which cytoskeletal proteins, chaperones, oxidoreductase, hydrolase and nucleic acid binding proteins were the major classes at both time points of infection (Fig. 4b). Even more interestingly, at 48 hpi we observed significant protein deimination of several members of the interferon (IFN)-induced protein with tetratricopeptide repeat (IFIT) family, such as IFIT1, IFIT2 and IFIT3, and of the IFN-inducible myxovirus resistance 1 (Mx1) gene product (Fig. 4a, left panel)—at 96 hpi, citrullination of these proteins was no longer detectable (Fig. 4a, right panel). Lastly, several heat shock proteins (HSPs) were found to be highly deiminated at either time point (Fig. 4a and Supplementary Data Set).
To validate the citrullinome findings, total proteins from mock or infected HFFs at 48 hpi were immunoprecipitated with the anti-citrulline F95 antibody and subjected to immunoblotting using antibodies against Mx1, IFIT1 and HSPH1. As shown in Supplementary Fig. 5, all three proteins were robustly deiminated following infection with HCMV.
IFITs are a family of antiviral RNA-binding proteins highly expressed during antiviral immune responses. In this regard, IFIT family members, known primarily for their antiviral activity against RNA viruses, have only recently been implicated in the innate immune response against DNA

viruses[23]. Specifically, Li and Swaminathan[24] have shown that human IFIT1, IFIT2 and IFIT3 proteins can suppress lytic replication of the Kaposi's sarcoma-associated herpesvirus (KSHV). Furthermore, Zhang *et al.* have demonstrated that IFIT1 overexpression significantly impairs HCMV replication in astrocytes, whereas IFIT1 knockdown sustains the viral cycle of HCMV [25].

The other IFN-inducible target of HCMV-induced deimination, Mx1, is a member of the dynamin-like large GTPase family involved in protection against negative-stranded RNA virus infection and several DNA viruses[26]. Interestingly, Mx2 has been recently shown to display antiviral activity against herpesviruses[27,28].

In order to gain further insight into the role of these genes during HCMV infection, we measured virus production in HCMV-infected HFFs after siRNA-mediated depletion of Mx1 and IFIT1—the latter having been shown to exert antiviral activity against HCMV only in fetal astrocytes. HFFs were transfected with specific siRNA, at 24 h post transfection (pt), infected with HCMV to induce interferon stimulated genes, and harvested at 48 hpi. Approximately 60% and 100% depletion of IFIT1 and Mx1, respectively, was achieved as confirmed by densitometric analysis (Supplementary Fig. 6). Both IFIT1 and Mx1 depletion led to 1-log higher levels of virus production compared to those observed in control siRNA-transfected cells (Fig. 4c), indicating that both Mx1 and IFIT1 strongly restrict HCMV infection.

In conclusion, our findings demonstrate that HCMV growth relies on PAD-mediated citrullination of host cell proteins such as IFIT1-3 and Mx1, suggesting that this PTM constitutes a potential druggable target for the design of new antivirals against HCMV and, more in general, herpesviruses. Finally, HCMV has been implicated in the pathogenesis of various degenerative diseases, such as autoimmune and inflammatory diseases, cardiovascular diseases and cancer, all characterized by a high degree of host protein citrullination. Overall, the unprecedented finding that HCMV-mediated host protein citrullination favors viral persistence provides groundbreaking insight into the mechanisms through which HCMV triggers the onset of the aforementioned diseases.

Methods

Cells and viruses. Human foreskin fibroblasts (HFFs, ATCC SCRC-1041™), African green monkey kidney cells (Vero) and human embryonic kidney 293 cells (HEK 293, Microbix Biosystems Inc.) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich, Milan, Italy) as previously described[29]. C8166 CD4+ lymphoblastoid T cells were kept in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% FCS (Sigma-Aldrich, Milan, Italy). HCMV strain Merlin was kindly provided by Gerhard Jahn and Klaus Hamprecht (University Hospital of Tübingen, Germany), and similarly to the HCMV laboratory strain AD169 (ATCC-VR538), was propagated and titrated on HFFs by standard plaque assay[30]. UV-inactivated Merlin was prepared using a double pulse of UV-B light (1.2 J/cm^2). AD169ΔIE1 was kindly provided by Thomas Stamminger (University of Ulm, Germany).
A clinical isolate of adenovirus was propagated in HEK 293 cells, whereas clinical isolates of HSV-1 and HSV-2 were grown in Vero cells and titrated by standard plaque assay[31]. HIV-1$_{IIIb}$ strain stock was prepared as previously described[32].
Recombinant adenoviral vectors (AdV) encoding HCMV IE2 (AdVIE2) and *Escherichia coli* β-galactosidase (AdVLacZ) have been previously described[29,30]. AdV-IE72 (AdVIE1) was provided by Dr. Timothy F. Kowalik (University of Massachusetts Medical School, Worcester, MA)[33]. Recombinant AdV stocks were generated, purified, and titrated as previously described[29,30,33]. For adenoviral transduction, HFFs were infected at ~80–90% confluence at an MOI of 10 PFU/cell. The total MOI was equalized to 10 with AdVLacZ.

Reagents and proteins. Recombinant human PAD2 and Cl-amidine were from Cayman Chemical (Ann Arbor, USA). Cycloheximide and Foscarnet were from Sigma-Aldrich (Milan, Italy).

***In vitro* antiviral assay.** HFFs, HEK 293 and Vero were incubated with increasing concentrations of Cl-amidine (0, 25, 50, 75, 100, or 200 μM) 1 h prior to being infected with HCMV, adenovirus

or HSV-1 or HSV-2 at an MOI of 0.1. Following virus adsorption (2 h at 37°C), cultures were maintained in medium containing the corresponding Cl-amidine and then incubated until control cultures displayed extensive cytopathology (7 days pi for HCMV, 6 days pi for adenovirus, and 48 hpi for HSV-1 and HSV-2). Thereafter, the cells and supernatants from the antiviral assay were harvested and disrupted by sonication. The extent of virus replication was then assessed by titrating the infectivity of supernatants as previously described[31,34].

Cell viability assay. To determine cell viability, HFFs, HEK 293, and Vero were exposed to increasing concentrations of Cl-amidine. After 6 days of incubation, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, as previously described[35]. The viability of the C8166 cells in presence of scalar concentrations of Cl-amidine was determined by trypan blue exclusion technique at day 7 pi.

HIV-1 infection and antiviral assay. HIV-1_{IIIb} (5 ng/ml of HIV-1 gag p24) was pre-incubated for 1 h at 37°C with increasing amount of Cl-amidine (0, 10, 25, 75, 100, or 200 µM) and then added to C8166 cells ($0.5x10^6$ cells/ml) for 2 h at 37°C. After three washes in 1X phosphate-buffered saline (PBS), cells were seeded at 5×10^5 cells/ml into fresh medium *plus* the same drug concentration used in the pre-incubation. The HIV-1 gag p24 amount was determined at day 7 post-infection in the culture supernatants with the HIV-1 p24 antigen ELISA kit (Biomerieux, Marcy-l'Étoile, France). Mock-infected C8166 cells were used as negative control. In parallel, HIV-1_{IIIb}-infected C8166 cells were treated with 5 µM tenofovir (NIBSC, London, UK) with the same procedure used for the drug treatment.

RNA isolation and quantitative nucleic acid analysis. Total RNA was extracted using the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) and 1 µg was retrotranscribed using the Revert-Aid H-Minus FirstStrand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, USA),

according to the manufacturer's instructions. Comparison of mRNA expression between samples (i.e. infected *vs* untreated) was performed by SYBR green-based RT-qPCR using Mx3000P apparatus (Stratagene, San Diego, USA), using the primers reported in Supplementary Table 1. To determine the number of viral DNA genomes *per* nanogram of cellular reference DNA (GAPDH gene), viral DNA levels were measured by quantitative qPCR on an Mx 3000 P apparatus (Stratagene, San Diego, USA). HCMV DNA copy numbers were normalized by dividing by the amount of human GAPDH gene amplified *per* reaction mixture. A standard curve of serially diluted genomic DNA mixed with an IE1-encoding plasmid (from 10^7 to 1 copy) was created in parallel with each analysis.

Western blot analysis. Whole-cell protein extracts were prepared and subjected to immunoblotting as previously described[36]. The following primary antibodies were used: anti-peptidyl-citrulline, clone F95 (1:500, MABN328; Merck Millipore, Burlington, USA), anti-PAD2 (1:1500, SML-ROI002-EX; Cosmo Bio, Eboli, Italy), anti-IFIT1 (1:1000, PA3-848; ThermoFischer, Waltham, USA), Anti-HSPH1 (1:1000, HPA028675; Sigma-Aldrich, Milan, Italy), anti-Mx1 (1:500, ab95926; Abcam, Cambridge, United Kingdom), anti-PADI6 (1:500, ab169416; Abcam, Cambridge, United Kingdom), anti-PADI1 (1:1000, ab24008; Abcam, Cambridge, United Kingdom), anti-PADI3 (1:500, ab172959; Abcam, Cambridge, United Kingdom), anti-PADI4 (1:500, ab128086; Abcam, Cambridge, United Kingdom), anti-α-tubulin (1:1000, 39527; Active Motif, La Hulpe, Belgium), IEA (1:1000, CH443; Virusys, Taneytown, USA), anti-ICP36 (1:1000, CA006; Virusys, Taneytown, USA), anti-pp28 (1:1000, CH19; Virusys, Taneytown, USA), anti-pp65 (1:1000, CA003; Virusys Taneytown, USA) . Immunocomplexes were detected using appropriate secondary antibodies conjugated with horseradish peroxidase (HRP) (ThermoFischer, Waltham, USA) and visualized by enhanced chemiluminescence (Super Signal West Pico; Thermo Fischer Scientific, Waltham, USA).

Detection of citrullination with the rhodamine-phenylglyoxal (Rh-PG) probe. Equal amounts of protein were diluted with trichloroacetic acid and incubated with Rh-PG as previously described[19]. The reaction was quenched with L-citrulline, washed with acetone and resuspended in 2X SDS loading dye for gel electrophoresis. Gels were imaged (excitation=532 nm, emission=580 nm) using a Biorad Chemidoc, stained with brilliant blue G-colloidal solution (Sigma-Aldrich, Milan, Italy).

Enzyme-linked immunosorbent assay activity. Plates (Nunc® 96 MaxiSorp™, Sigma-Aldrich, Milan, Italy) were coated with 4 µg/mL of calf thymus histone 3 (Sigma-Aldrich, Milan, Italy) in coating buffer (100 mM Na_2CO_3, 100 mM $NaHCO_3$, pH 9.6) and incubated at 4°C overnight. Subsequently, they were washed with 1X TBST (Tris-buffered saline, 0.05 % Tween20). Then, 50 µg protein lysates from HCMV-infected HFFs at 15, 24, 48, or 72 hpi or from uninfected-mock, in the presence or absence of 100 µM Cl-amidine or with the same volume of the vehicle as negative control, were diluted in calcium-free PAD reaction buffer (2 mM DTT, 50 mM NaCl,100 mM Tris, pH 7.57). As positive control, increasing concentrations of 2.5, 5 and 10 mU of recombinant PAD2 (Cayman Chemical, Ann Arbor, USA), diluted in PAD reaction buffer containing 10 mM $CaCl_2$, were applied. The samples were incubated for 20 h at 37°C. Plates were blocked in 100 µL/well of blocking solution (2% BSA in 1X PBST) at room temperature for 1 h. After incubation with blocking buffer, an anti-human citrullinated histone 3 primary antibody (1:2000, ab5103; Abcam, Cambridge, United Kingdom) and anti-rabbit IgG-Fc HRP-conjugated secondary antibody were used to detect citrullinated proteins (1:7000, GE Healthcare Europe GmbH, Freiburg, Germany). Subsequently, 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma-Aldrich, Milan, Italy) was added to each well. The amplification of the signal by HRP was given by the blue color obtained after 30 min incubation, which changed to yellow once stopped by the addition of 1 N HCl. The optical density (OD) was measured at 450 nm to 620 nm using a VICTOR3 Multilabel Reader (PerkinElmer)[37,38].

Construction of promoter reporter plasmids. The 5'-flanking region of *PADI2* was generated by PCR using Q5 High-Fidelity DNA polymerase (New England Biolabs, Ipswich, USA), pLightSwitch_Prom *PADI2* (Active Motif, La Hulpe, Belgium), as the template, and the primers listed in Supplementary Table 2. The thermocycler settings consisted of 30 s incubation at 98°C, followed by 35 cycles at 98°C for 10 s, 30 s at the predicted melting temperature and 30 s at 72°C, and a final extension for 2 min at 72°C. The 5'-flanking region of *PADI4* was amplified by PCR using human genomic DNA from HFFs, as the template, and the primers listed in Supplementary Table 2. The PCR condition was an initial denaturation for 2 min at 95°C, 35 cycles (95°C for 30 sec, 56°C for 30 sec, and 72°C for 4 min) and a final extension at 72°C for 8 min. The resulting amplification products were digested with XhoI and HindIII and cloned into the pGL4.20[luc2/Puro] Vector, which encodes the luciferase reporter gene luc2 (Photinus pyralis), but no regulatory elements. All of the constructs were prepared using the PureYield Plasmid Miniprep System (Promega, Madison, USA) and verified by restriction mapping and complete sequencing.

Luciferase assay. HFFs were electroporated using a Micro-Porator MP-100 (Thermo Fischer Scientific, Waltham, USA), according to the manufacturer's instructions (a single 1300 V pulse, 30 ms pulse width). Briefly, 500 ng of each construct were used every 2×10^5 cells, which were plated in 24-well tissue culture clusters at a density of 2×10^5 cells/well. To correct for transfection efficiency, all cells were co-transfected with the pRL-SV40 vector, which contained the Renilla luciferase gene driven by the SV40 promoter. After 24 h, cells were infected with HCMV, UV-HCMV or mock (MOI of 1 PFU/ml). At 24 hpi, firefly and Renilla luciferase activities were measured using the Dual-Luciferase reporter assay system kit (Promega, Madison, USA) and a Lumino luminometer (Stratec Biomedical Systems, Birkenfeld, Germany). Firefly luciferase activity from the luciferase reporter vector was normalized to the Renilla luciferase activity from

the pRL-SV40 vector. Values were expressed as the ratio of relative light units (RLU) measured for firefly luciferase activity to the RLU measured for that of the Renilla luciferase.

CRISPR-Cas9 vector constructs. The CRISPR/Cas9 system was employed to generate specific gene knockouts in HFFs. Briefly, a lentiviral CRISPR/Cas9 vector that encodes a codon-optimized nuclear-localized Cas9 gene N-terminally fused to the puromycin resistance gene *via* a T2A ribosome-skipping sequence was employed. This vector contains a human U6 promoter driving expression of a guideRNA (gRNA) consisting of a gene-specific CRISPR RNA (crRNA) fused to the trans-activating crRNA (tracrRNA) and a terminator sequence[39]. gRNA sequences are reported in Supplementary Table 3. An empty vector carrying no gRNA was used as negative control (WT cell line). All constructs were verified by Sanger sequencing.

Lentivirus production and transduction of HFFs with lentiviral CRISPR/Cas9. Recombinant lentiviruses were packaged in HEK 293T cells by cotransfection of the 3rd Generation Packaging System Mix (kindly provided by A. Follenzi, University of Eastern Piedmont, Novara) with the above mentioned vectors to produce viral particles using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, USA). Viral supernatants were harvested after 72 h and used to transduce HFFs by infection in the presence of 8 mg/ml polybrene. Transduced cells were selected with puromycin (1 μg/ml) over the course of 14-day post-transduction. After selection, successful knockout was confirmed using immunoblotting and TIDE analysis.

TIDE. After selection, successful knockout was confirmed using immunoblotting. Additionally, indel frequencies were quantified using TIDE[40]. Genomic DNA was extracted, and PCR amplicons spanning the single guide RNA (sgRNA) target site were generated. The purified PCR products were then Sanger sequenced, and indel frequencies were quantified using the TIDE software (http://tide.nki.nl) (Supplementary Fig. 4). A reference sequence (wild-type cells) was used as a

control. Genomic DNA was isolated from 1×10^6 cells using the ISOLATE II Genomic DNA Kit (Bioline Meridian Biosciences, Paris, France). PCR reactions were carried out with 50 ng genomic DNA and Q5 High-Fidelity DNA polymerase according to manufacture instructions. PCR conditions were 30 s at 98°C (1 cycle), followed by 10 s at 98°C, 30 s at 5°C and 30 s at 72°C (35 cycles). The PCR products were purified using the GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermo Fischer Scientific, Waltham, USA). The primer pairs spanning the target site are reported in Supplementary Table 4. The purified PCR products were then Sanger sequenced by TubeSeq Service (Eurofins Genomics, Ebersberg, Germany), and indel frequencies were quantified using the TIDE software (http://tide.nki.nl). A reference sequence (WT) was used as a control.

Pull down. Uninfected or HCMV-infected cells (MOI of 1 PFU/ml) were washed with 1X PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris pH 7.4; 150 mM NaCl; 1 mM EDTA; 1% nonidet P-40; 0.1% SDS; 0.5% deoxycholate; protease inhibitors). Proteins (200 μg) were then incubated with 2 μg of F95 antibody or with an isotype antibody as negative control (62-6820; Thermo Fischer Scientific, Waltham, USA) for 1 h at room temperature with rotation followed by overnight incubation at 4°C with protein G-Sepharose (Sigma-Aldrich, Milan, Italy). Immune complexes were collected by centrifugation and washed with RIPA buffer. The Sepharose beads were pelleted and washed three times with RIPA buffer, resuspended in reducing sample buffer (50 mM Tris pH 6.8; 10% glycerol; 2% SDS; 1% 2- mercaptoethanol), boiled for 5 min and resolved on a SDS-PAGE gel to assess protein binding by immunoblotting.

Mass spectrometry sample preparation. Sample preparation in technical triplicates followed the procedure outlined in Tilvawala et al. 2018[41]. Equal amounts of cell lysates from each experimental group (300 μg) were diluted in buffer (100 mM HEPES pH 7.6) to a final concentration of 1 μg/μL and incubated with 20% trichloroacetic acid (TCA) and 0.5 mM biotin-PG[42] for 30 min at 37 °C. Labeled proteomes were precipitated on ice for 30 min. Samples were pelleted through tabletop

centrifugation (15,000 rpm, 15 min) at 4 °C. The supernatants were discarded, and the pellets were washed with cold acetone (300 μL). After drying for 5 min, the pellets were resuspended in 1.2% SDS in PBS by bath sonication and heating. Samples were then transferred to 15 mL screw cap tubes and diluted in 1X PBS with 0.2% SDS. Samples were incubated with streptavidin agarose slurry (Sigma Aldrich, 170 μL) overnight at 4 °C and for an additional 3 h at 25 °C. After discarding the flow through, the streptavidin beads were washed with 0.2% SDS in PBS (5 mL) for 10 min at 25 °C. The beads were then washed three times with 1X PBS (5 mL) and three times with water (5mL) in order to remove any unbound proteins. Beads were then transferred to a screw cap microcentrifuge tube and heated in 1X PBS with 500 μL 6 M urea and 10 mM DTT (65 °C, 20 min). Proteins bound to the beads were then alkylated with iodoacetamide (20 mM, 37 °C for 30 min). The beads were successively pelleted by centrifugation (1,400 x *g* for 3 min) and the supernatant was removed. The pellet was resuspended in a premixed solution of 2 M urea, 1 mM $CaCl_2$ and 2 μg Trypsin Gold (Promega) in PBS. These were shaken overnight at 37 °C. The supernatant was collected and the beads were washed twice with water (50 μL), each time collecting the water. The fractions were combined, acidified with formic acid (5% final concentration) and stored at -20 °C until use.

Mass spectrometry. Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analysis was performed with an LTQ-Orbitrap Discovery mass spectrometer (ThermoFisher) coupled to an Easy-nLC HPLC (ThermoFisher). Samples were pressure loaded onto a 250 μm fused-silica capillary hand packed with 4 cm Aqua C18 reverse phase resin (Phenomenex). Samples were separated on a hand packed 100 μm fused-silica capillary column with a 5 μm tip packed with 10 cm Aqua C18 reverse phase resin (Phenomenex). Peptides were eluted using a 10-h gradient of 0-100% Buffer B in Buffer A (Buffer A: 95% water, 5% acetonitrile, 0.1% formic acid; Buffer B: 20% water, 80% acetonitrile, 0.1% formic acid). The flow rate through the column was set to ~400

nL/min, and the spray voltage was set to 2.5 kV. One full MS scan (FTMS) was followed by 7 data-dependent MS2 scans (ITMS) of the n^{th} most abundant ions. The tandem MS data were searched by the SEQUEST algorithm using a concatenated target/decoy variant of the human UniProt database. A static modification of +57.02146 on cysteine was specified to account for alkylation by iodoacetamide. SEQUEST output files were filtered using DTASelect 2.0.

GO Protein class analysis. PANTHER (http://www.pantherdb.org) gene list analysis was used to functionally classify citrullinated proteins based on protein classes based on the UniProt ID code[43].

siRNA-mediated knockdown. HFFs were transiently transfected with a MicroPorator (Digital Bio Pharm, London, Great Britain) according to the manufacturer's instructions (1200 V, 30 ms pulse width, one impulse) with a pool of small interfering RNAs (Qiagen, Hilden, Germany) targeting Mx1 (siMx1, FlexiTube siRNAs cat. N.: SI02781093, SI05459538, SI04435963, SI04435956), or control siRNA (siCTRL, 1027292) as negative control. For siRNA-mediated knockdown of IFIT1, siRNA approach was performed according to Pichlmair et al. (**siIFIT1_1**: CTCCTTGGGTCGTTCTACAAA; **siIFIT1_2**: TACATGGGAGTTATCCATTGA)[44].

Statistical analysis. All statistical tests were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, USA). The data were presented as means ± standard deviations (SD). Statistical significance was determined by using one-way or two-way analysis of variance (ANOVA) with Bonferroni's and Tukey's post-tests, as appropriate. Differences were considered statistically significant for $P<0.05$ ($P<0.05$*; $P<0.01$**; $P<0.001$***).

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Author contributions

G.G., F.G., S.P. and S.L. conceived and designed the experiments. G.G., F.G., S.P., V.D.O., M.B. performed the experiments and analyzed the data. A.J. S., P.R.T. and E.W. carried out the LC-MS/MS analyses. A.J.S. and R.J. L. supported the generation of the CRISPR-Cas9 KO cell lines. T.S. supported the design of the experiments with AD169ΔIE1. All authors contributed to writing this manuscript.

Competing interests

The authors declare the following competing financial interest(s): P.R.T. founded Padlock Therapeutics and is entitled to payments from Bristol Myers Squibb if certain milestones are met. P.R.T. is a consultant for Celgene and Disarm Therapeutics. Italian Patent No. 102011596547852 registered in October 20th, 2019 (Notifica domanda di brevetto italiana "PAD2 PER USO NELLA PREVENZIONE E/O TRATTAMENTO O DIAGNOSI DI INFEZIONI DA VIRUS DELLA FAMIGLIA HERPESVIRIDAE").

Figures

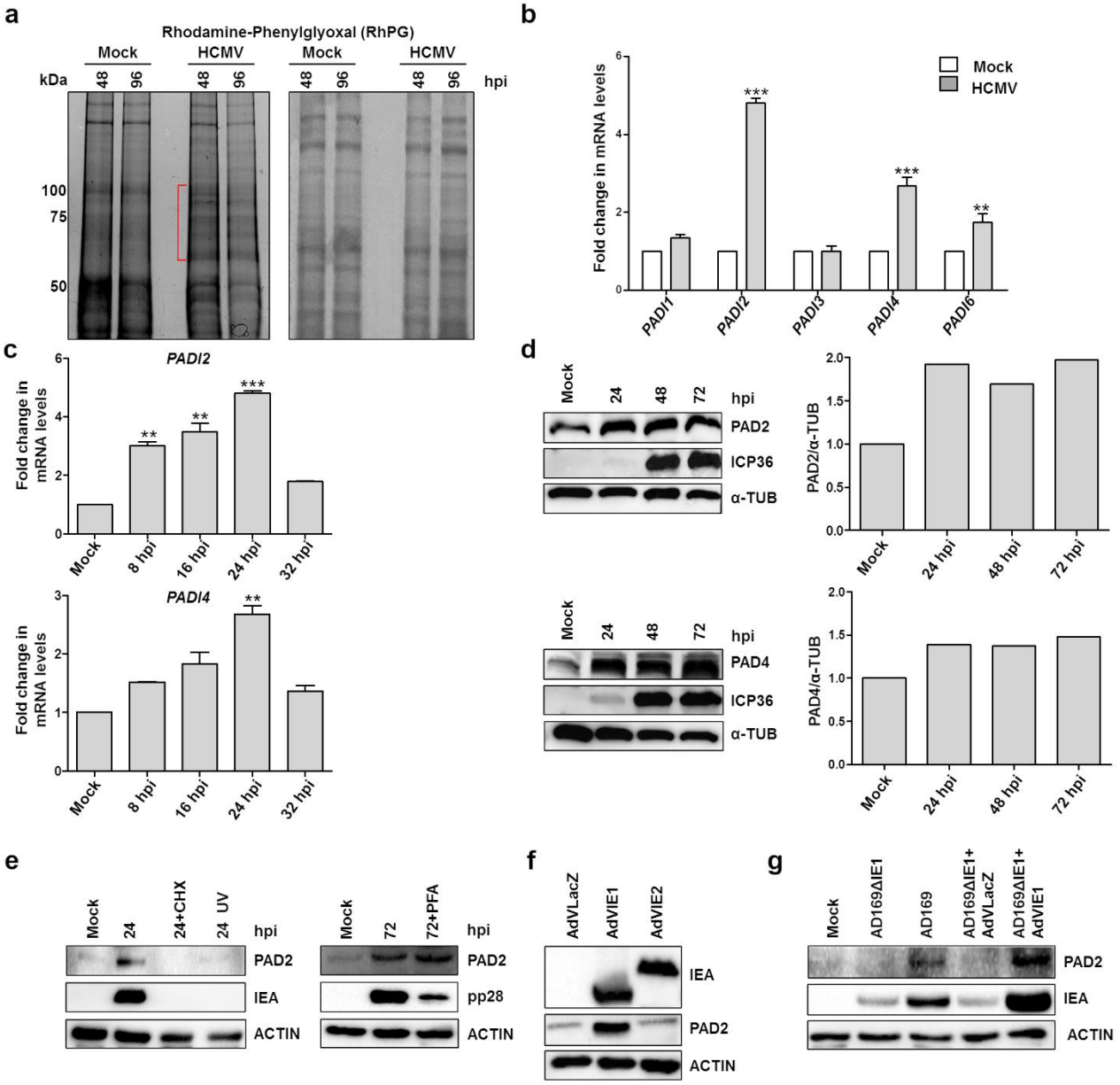


Fig. 1 HCMV infection induces protein citrullination. a, Protein lysates from HFFs infected with HCMV (MOI 1 PFU/cell) at 48 and 96 h post infection (hpi) or from uninfected HFFs (mock) were exposed to an Rh-PG citrulline-specific probe (left panel) and subjected to gel electrophoresis to detect citrullinated proteins. Equal loading was assessed by Coomassie blue staining (right panel). The red bracket highlights the increased number of citrullinated proteins ranging from 60 to 100 kDa at 48 hpi. **b,** mRNA expression levels of *PADI* isoforms by qRT-PCR of HCMV-infected (24 hpi) *vs* uninfected (mock) HFFs were normalized to the housekeeping gene glyceraldehyde-3-

phosphate dehydrogenase GAPDH and expressed as mean fold change ± SD over mock-infected cells (*n* = 3 independent determinations;**, P< 0.01; ***, P< 0.001; two-way ANOVA followed by Bonferroni's post-tests, for comparison of infected versus mock cells). **c,** *PADI2* and *PADI4* mRNA levels in HCMV-infected HFFs at the indicated time points (hpi) were normalized to GAPDH mRNA and expressed as mean fold change ± SD over mock-infected cells (*n* = 3 independent determinations; **, P< 0.01; ***, P< 0.001; one-way ANOVA followed by Bonferroni's post-tests, for comparison of infected versus mock cells). **d,** Western blot analysis of protein lysates from uninfected (mock) or infected HFFs using antibodies against PAD2, PAD4, ICP36 or α-tubulin. One representative blot and densitometric analysis is shown of representative of three independent experiments. Values are expressed as fold change in PAD2 and PAD4 expression normalized to α-tubulin. **e,** Western blot analysis of protein lysates from uninfected (mock) or infected HFFs with HCMV wild-type (HCMV) or UV-inactivated HCMV (UV), treated with 150 μg/ml CHX (left panel) at 24 hpi, or with 250 μM PFA (right panel) at 72 hpi or left untreated. Analysis was performed using antibodies against PAD2, IEA, pp28 or actin. One representative blot of three independent experiments is shown. **f**, Western blot analysis of protein lysates from uninfected (mock), infected HFFs for 48h with AdVIE1, AdVIE2 or AdVLacZ (MOI=10) using antibodies against IEA, PAD2 or actin. One representative blot of three independent experiments is shown. **g**, Western blot analysis of protein lysates from HFFs infected with wtAD169 HCMV or AD169ΔIE1 (MOI 1 PFU/cell), the latter complemented with AdVLacZ or AdVIE1 (MOI 10 PFU/cell) at 48 hpi or from uninfected HFFs (mock) using antibodies against IEA, PAD2 or actin. One representative blot of three independent experiments is shown.

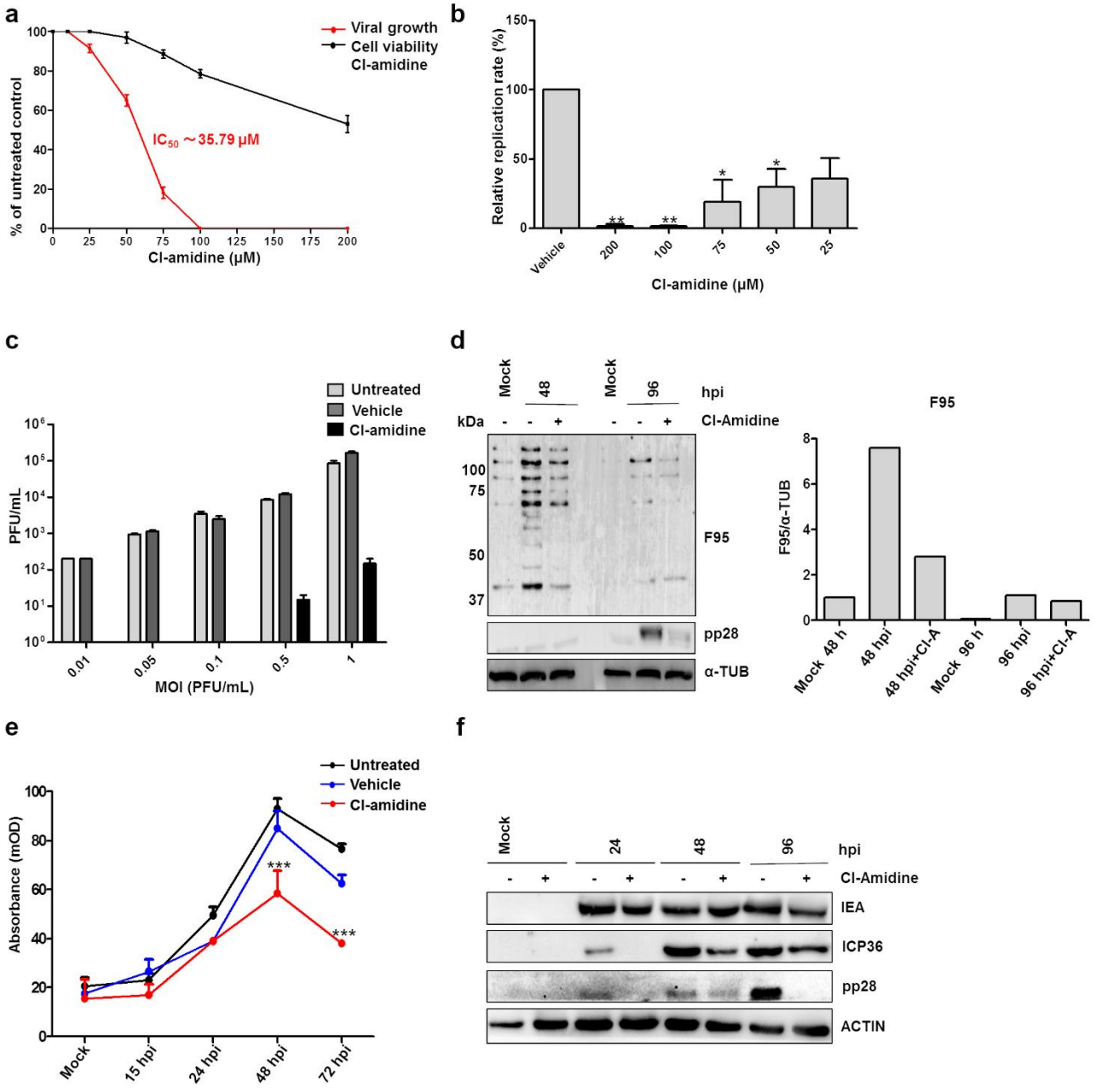


Fig. 2 Cl-amidine blocks HCMV replication. a, HFFs were infected with HCMV (MOI 0.1 PFU/cell) and then treated with increasing concentrations of Cl-amidine, which were given 1 h prior to virus adsorption and kept throughout the whole experiment. At 144 hpi, viral plaques were microscopically counted and expressed as percentage relative to untreated controls. The number of plaques is plotted as a function of Cl-amidine concentration. The concentrations resulting in 50% plaque formation (IC_{50}) reduction are represented by the red line. The number of viable cells was determined for each Cl-amidine concentration using the MTT method (black line). Values are expressed as means ± SD (error bars) of three independent experiments. **b,** To determine the

number of viral DNA genomes in HCMV-infected HFFs, viral DNA was isolated at 144 hpi and analyzed by qRT-PCR using primers amplifying a segment of the IE1 gene. GAPDH was used to normalize HCMV genome counts. Values are expressed as mean ± SD of three independent experiments (*P<0.05, **P<0.01, one-way ANOVA followed by Bonferroni's post test). **c,** HFFs were infected with HCMV at increasing MOIs (0.01-1 PFU/cell) and then treated with 100 µM Cl-amidine or vehicle. Viral supernatants were collected at 144 hpi and analyzed by standard plaque assay. Values are expressed as mean ± SD of three independent experiments. **d,** Protein lysates from uninfected (mock) or infected HFFs (48 and 96 hpi) with (+) or without (-) 100 µM Cl-amidine were subjected to immunoblotting using the anti-peptidylcitrulline F95 antibody to detect citrullinated proteins, anti-pp28 to assess HCMV infection, or anti-α-tubulin to show equal loading. The densitometric analysis shown is representative of three independent experiments. Densitometry values of F95 are normalized to those of α-tubulin. **e,** PAD enzymatic activity assay. Histone H3 was immobilized on a 96-well microtiter plate and incubated with protein lysates from HCMV-infected HFFs or uninfected-mock at the indicated time points, in the presence (red line) or absence (untreated or vehicle alone, black and blue line, respectively) of Cl-amidine. The conversion of peptidylarginine to peptidylcitrulline was detected with an anti-H3 citrulline antibody. Detection of the bound antibodies was performed by ELISA. Values are expressed as means ± SD (error bars) of three independent experiments (***, P<0.001; two-way ANOVA followed by Bonferroni's post-tests). **f,** Protein lysates from uninfected (mock) or infected HFFs (24, 48 or 72 hpi) at an MOI of 1 PFU/cell treated with or without Cl-amidine (+) or vehicle (-) were analyzed by immunoblotting for viral expression (IEA, ICP36, and pp28) and normalized to α-tubulin.

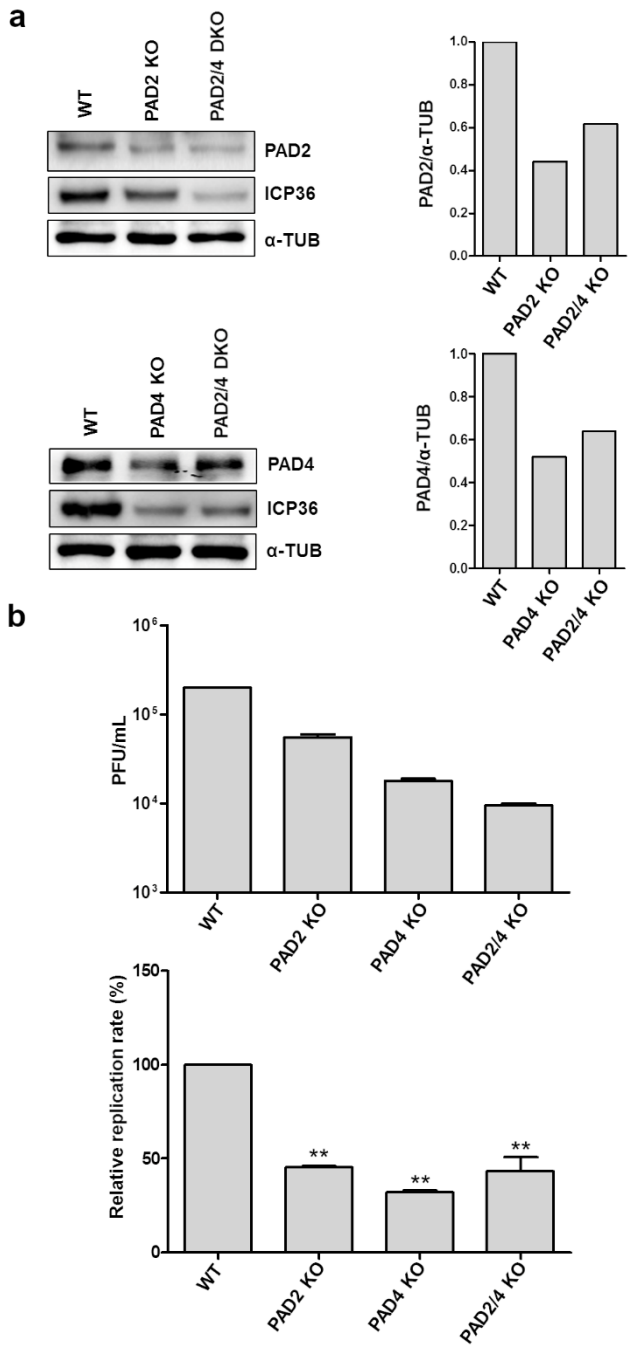


Fig. 3 Effect of PAD2 and PAD4 gene knockout on HCMV replication. a, Knockout (KO) gene variants in HFFs for PAD2 (PAD2 KO), PAD4 (PAD4 KO) or both PAD2 and PAD4 KO (PAD2/4 DKO) were generated using CRISPR-Cas9 technology. The efficiency of PAD2 and PAD4 protein depletion at 48 hpi was assessed by immunoblotting using antibodies against PAD2, 4, ICP36 to assess HCMV infection or α-tubulin for equal loading. The Western blot shown along with its densitometric analysis is representative of three independent experiments. Values are expressed as fold change in PAD2 and 4 expression normalized to α-tubulin. **b,** (upper panel) HFFs KO cells

were infected with HCMV at an MOI of 0.1 PFU/cell. Viral supernatants were collected at the indicated time points and analyzed by standard plaque assay. Values are expressed as means ± SD. (lower panel) To determine the number of viral DNA genomes in HCMV-infected HFFs KO cells (MOI 0.1), viral DNA was isolated at 144 hpi and analyzed by qRT-PCR using primers amplifying a segment of the IE1 gene. GAPDH was used to normalize HCMV genome counts. Values are expressed as mean ± SD of three independent experiments (**P<0.01, one-way ANOVA followed by Bonferroni's post test).

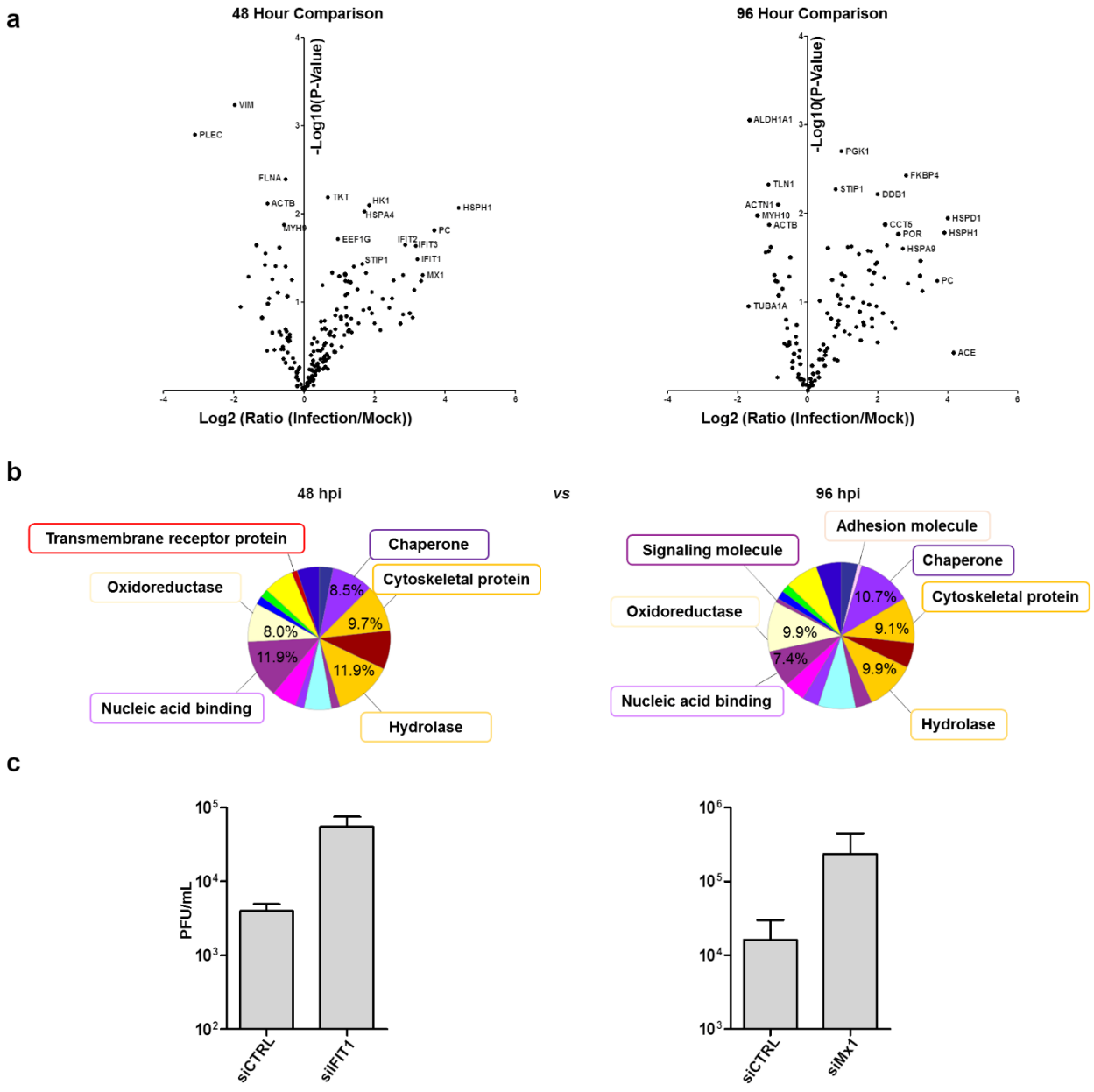
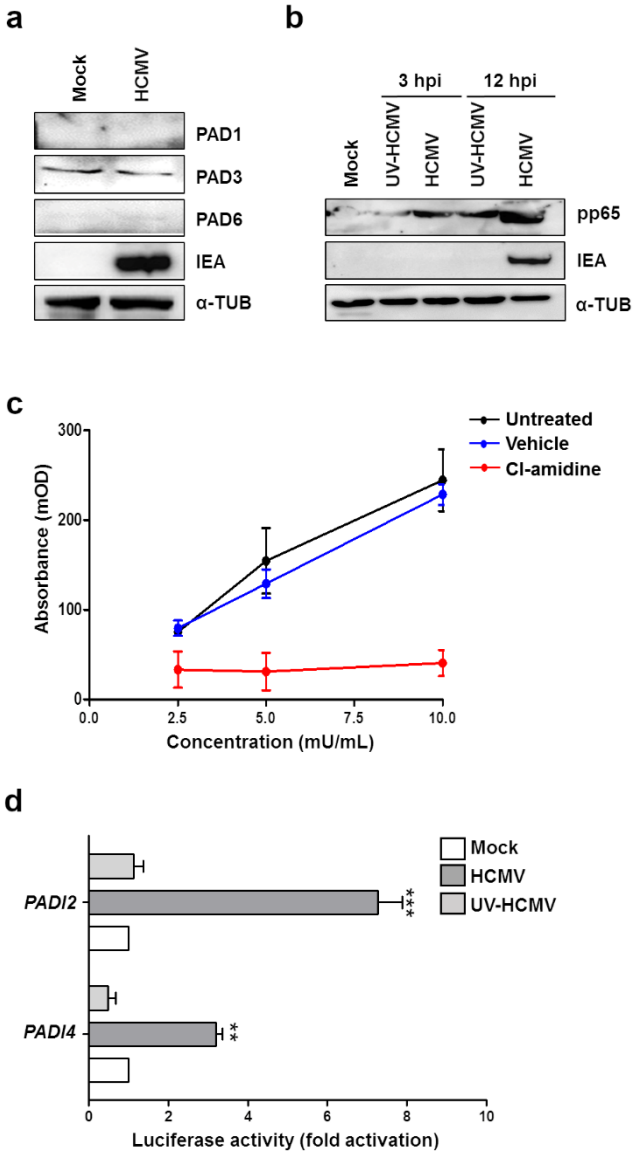


Fig. 4 Characterization of the citrullinated proteome (citrullinome) of HCMV infected cells. a, Volcano plot depicting the citrullinome of infected cells *vs* mock-infected cells at 48 hpi (left panel) and 96 hpi (right panel). Cell lysates from uninfected (mock) or HCMV-infected HFFs (MOI 1) were exposed to a biotin-PG to isolate citrullinated proteins on streptavidin agarose. Bound proteins were then subjected to on-bead tryptic digestion and analyzed by LC-MS/MSS—in the graph, every identified citrullinated protein corresponds to a dot. The x-axis represents the ratio of citrullination between mock and infected cells at the indicated time points, while the y-axis indicates the statistical significance. Both variables were plotted on a logarithmic scale (*n*=3). **b,** Pie charts show the

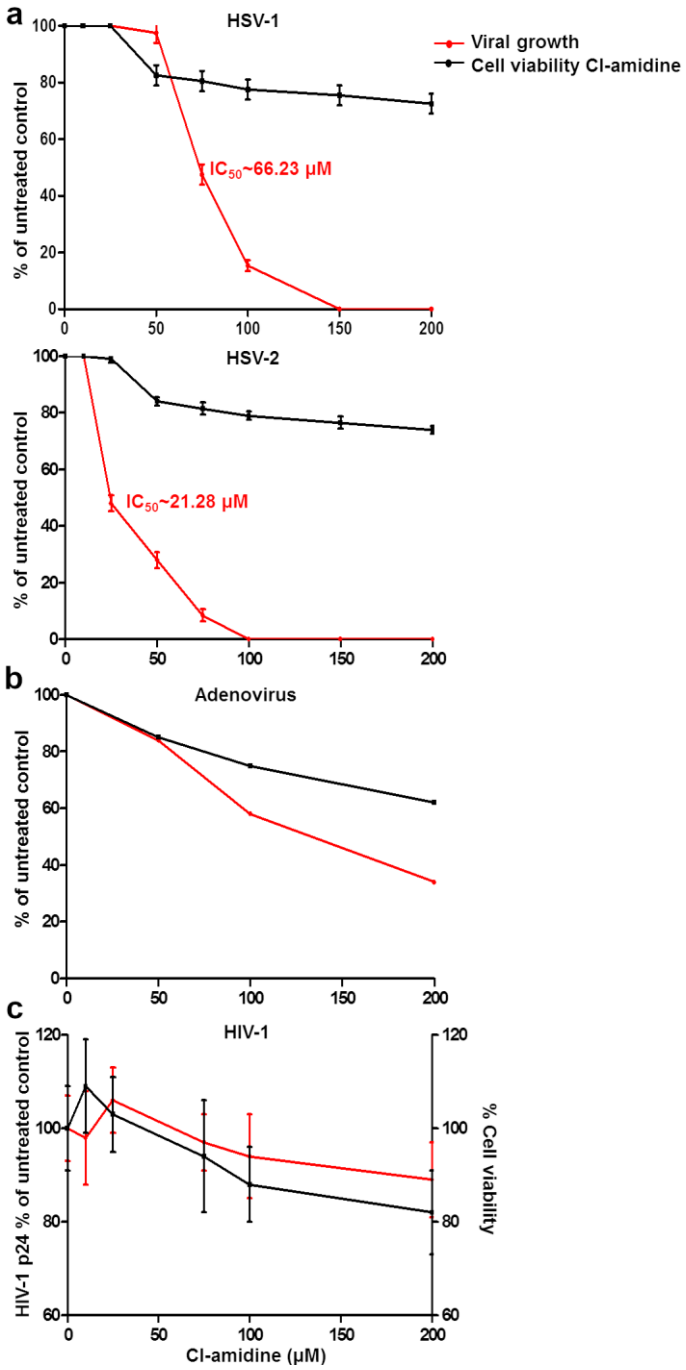
classification of citrullinated proteins from 48 hpi (left) and 96 hpi (right) based on protein classes (Panther software). **c,** HFFs were silenced for IFIT1 and Mx1 or with control siRNA using specific siRNAs (siIFIT1, siMx1 and siCTRL, respectively). At 24 hpt, cells were infected with HCMV at an MOI of 0.1 PFU/cell. Viral supernatants were collected at 144 hpi and analyzed by standard plaque assay. Values are expressed as means ± SD of three independent

SUPPLEMENTARY INFORMATION



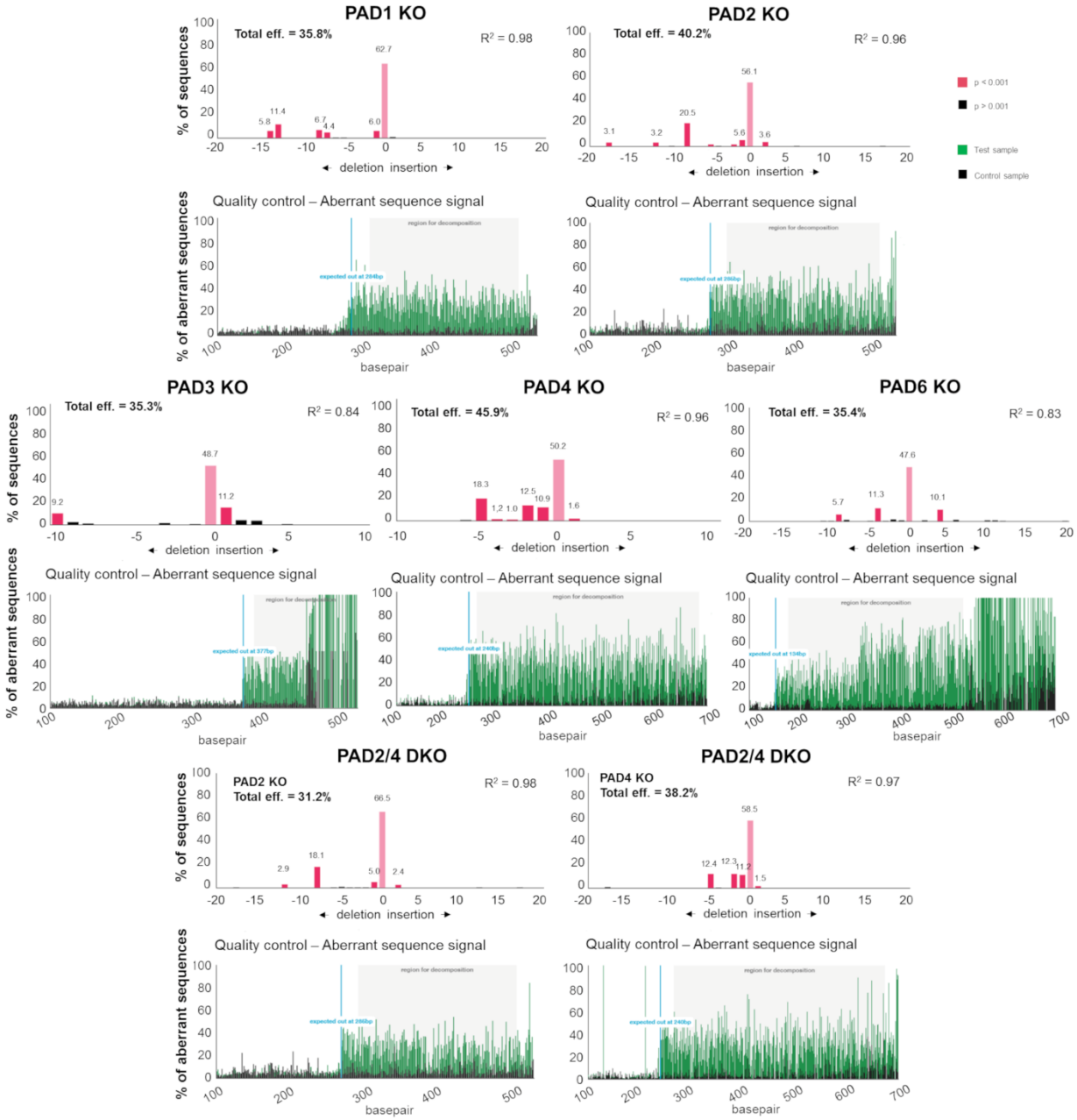
Supplementary Fig. 1. **PAD1, 3 and 6 protein expression during HCMV infection. a,** Protein lysates from uninfected (mock) or infected HFFs at 48 hpi were subjected to immunoblotting using antibodies against PAD1, PAD3, PAD6, IEA or α-tubulin (α-TUB). The Western blot shown is representative of three independent experiments. **b, UV-inactivated HCMV does not replicate.** Protein lysates from uninfected (mock) or infected HFFs (at 3 and 12 hpi) with either wild-type (HCMV) or UV-inactivated HCMV (UV-HCMV) were subjected to immunoblotting using

antibodies against IEA, pp65 or α-tubulin (α-TUB). **c, PAD enzymatic activity assay.** Histone H3 was immobilized on a 96-well microtiter plate and incubated with increasing concentration of recombinant human PAD2 in the presence (red line) or absence (untreated or vehicle alone, black and blue line, respectively) of Cl-amidine. The conversion of peptidylarginine to peptidylcitrulline was detected by means of an anti-H3 citrulline antibody. Bound antibodies were detected and visualized by ELISA. Values are expressed as means ± SD (error bars) of three independent experiments. **d, *PADI2* and *PADI4* promoter activation upon HCMV infection.** HFFs were transiently electroporated with luciferase plasmids encoding the wild-type *PADI2* promoter region (*PADI2*) or *PADI4* promoter region (*PADI4*) or pRL-SV40 as control and. Twenty-four hours later, the cells were mock-infected or infected with HCMV or UV-inactivated HCMV at an MOI of 1. At 24 hpi, firefly and Renilla luciferase activities were measured. Luciferase activity in whole-cell lysates was normalized to Renilla luciferase activity and expressed as relative light units (RLU) (right panel). Results are shown as a mean ± SD fold change (*, $P<0.05$; ***, $P<0.001$; one-way ANOVA followed by Bonferroni's post-tests).

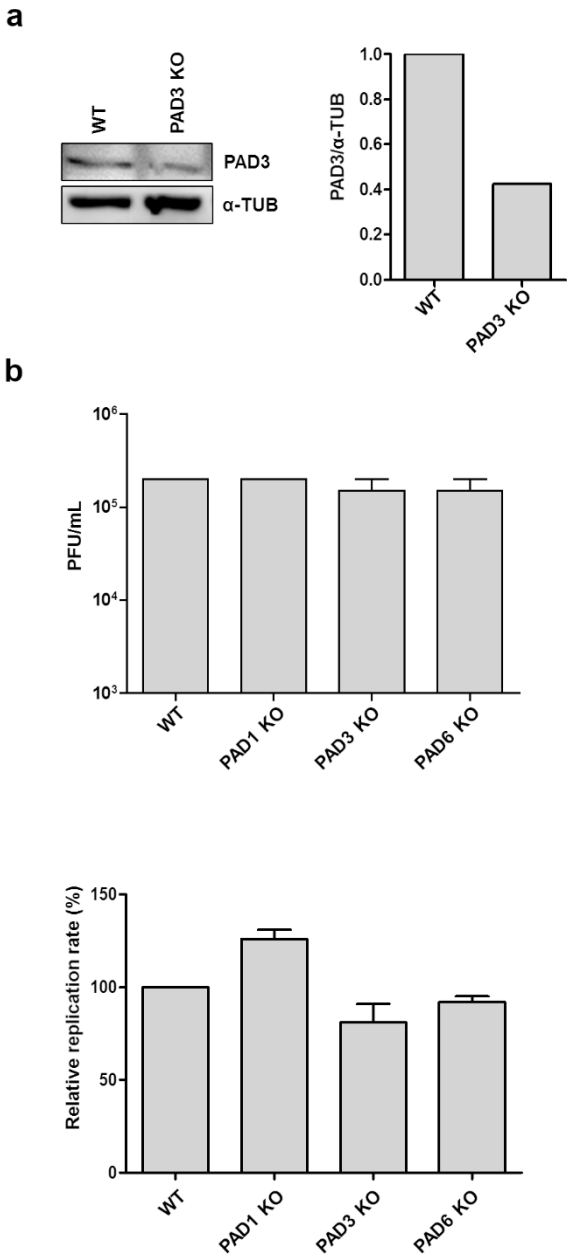


Supplementary Fig. 2. Antiviral activity of Cl-amidine against HSV-1 and HSV-2 replication. **a,** VERO cells were infected with HSV-1 (upper panel) or HSV-2 (lower panel) (MOI=0.1) and then treated with increasing concentrations of Cl-amidine, which were given 1 h prior to virus adsorption and kept throughout the whole experiment. At 48 hpi, viral plaques were microscopically counted and the mean plaque count for each drug concentration was expressed as percentage relative to the mean count of the untreated control. The number of plaques is plotted as a function of Cl-amidine concentration. The concentrations achieving 50% plaque formation (IC_{50})

reduction are shown (red line). To determine cell viability, the number of viable cells for each Cl-amidine concentration was determined using the MTT assay (black line). Values are expressed as means ± SD (error bars) of three independent experiments. **b,** Cl-amidine does not inhibit adenovirus and HIV-1 replication. HEK 293 cells were infected with a clinical isolate of adenovirus (MOI=0.1) and then treated with increasing concentrations of Cl-amidine as described above. At 144 hpi, viral plaques were microscopically counted, and the mean plaque count for each drug concentration was expressed as percentage of the mean count relative to the untreated control. The number of plaques is plotted as a function of Cl-amidine concentration (red line). To determine cell viability, the number of viable cells for each Cl-amidine concentration was determined using the MTT assay (black line). Values are expressed as means ± SD (error bars) of three independent experiments. **c,** HIV-1_{IIIb} strain (5 ng/ml of HIV-1 gag p24) was incubated for 1 h at 37°C in RPMI 1640 medium with serial concentrations of Cl-amidine (0, 10, 25, 75, 100, or 200 μM). Successively, these Cl-amidine-containing solutions were used to grow C8166 lymphoblastoid CD4+ T cells for 2 h at 37°C. HIV-1 replication was analyzed by HIV-1 p24 ELISA assay on cell culture supernatants at day 7 post-infection. Values are expressed as the means ± SD (error bars) of the level of HIV-1 gag p24 relative to untreated controls (set to 100%). Three independent experiments in duplicate were performed (red line). To determine cell viability, the number of viable cells for each Cl-amidine concentration was determined using trypan blue exclusion. Values are expressed as means ± SD of viable cells relative to untreated controls (set to 100%) obtained from three independent experiments each performed in duplicate (black line).

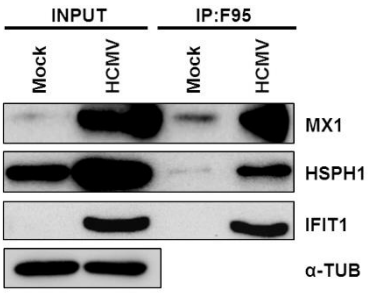


Supplementary Fig. 3. Assessment of genome editing in PAD1, PAD2, PAD3, PAD4, PAD6 KO and PAD2/PAD4 DKO cell lines. Successful genome editing was assessed by TIDE analysis. To this end, genomic DNA was extracted, and PCR amplicons spanning the single guide RNA (sgRNA) target site were generated. The purified PCR products were then Sanger sequenced, and indel frequencies were quantified by TIDE software. The overall cutting efficiency for each gene is between 35 and 45%, meaning that over 35% of the cells carry an indel (insertion or deletion) in the targeted gene leading to a deletion, insertion or a frameshift mutation in the reading frame, thereby rendering the gene of interest nonfunctional.

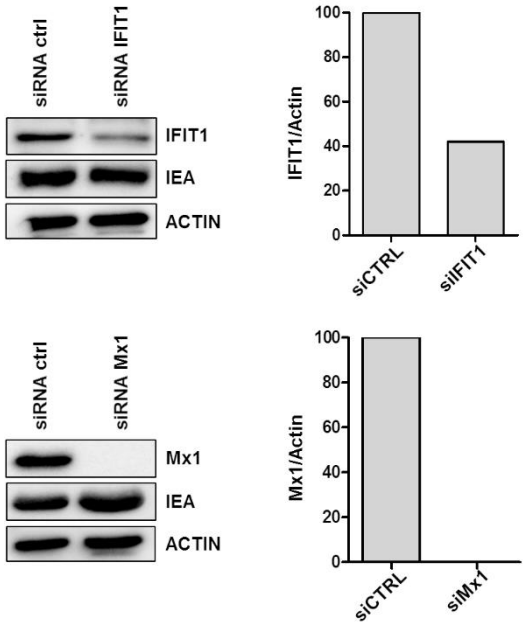


Supplementary Fig. 4. Effect of PAD1, PAD3 and PAD6 gene knockout on HCMV replication. a, Knockout (KO) gene variants in HFFs for PAD1 (PAD1 KO), PAD3 (PAD3 KO) and PAD6 (PAD6 KO) were generated using CRISPR-Cas9 technology. The efficiency of PAD3 protein depletion was assayed by Western blot analysis for PAD3, using an anti-α-tubulin (α-TUB) antibody as loading control. The Western blot shown along with its densitometric analysis is representative of three independent experiments. **b,** (upper panel) HFFs depleted of PAD1, PAD3, PAD6 were infected with HCMV at an MOI of 0.1. The extent of virus replication was measured at

the indicated time points by standard plaque assay. Values are expressed as means ± SD (error bars). (lower panel) To determine the number of viral DNA genomes in HCMV-infected HFFs KO cells (MOI 0.1), viral DNA was isolated at 144 hpi and analyzed by qRT-PCR using primers amplifying a segment of the IE1 gene. GAPDH was used to normalize HCMV genome counts. Values are expressed as mean ± SD (error bars) of three independent experiments.



Supplementary Fig. 5. HCMV infection triggers citrullination of IFIT1, MX-1 and HSPH1 proteins. Immunoprecipitation (IP) of total cell extracts (INPUT) from mock and infected HFFs at 48 hpi using the anti-peptidylcitrulline antibody F95 or an IgM non-correlated antibody, as control (data not shown). The IP complexes were analyzed by Western blotting using antibodies against Mx1, HSPH1 and IFIT1. Equal loading was assessed by α-tubulin immunoblotting (α-TUB). The blot shown is representative of three independent experiments.



Supplementary Fig. 6. Silencing of IFIT1 and MX-1. HFFs were transiently electroporated with specific siRNA against IFIT1 or Mx1 (siIFIT1, siMx1) or control (siCTRL). After 24 h, transfected cells were mock-infected or infected with HCMV at an MOI of 1. At 48 hpi, cells were harvested, and the efficiency of IFIT1 and Mx1 protein silencing was assayed by Western blot analysis using antibodies against IFIT1, Mx1, IEA and actin as loading control. The blot shown along with its densitometric analysis is representative of three independent experiments. Values are expressed as fold change in IFIT1 and Mx1 expression normalized to actin.

SUPPLEMENTARY TABLE

Supplementary Table 1. Oligonucleotide primer sequences for qPCR

GENE OF INTEREST	SEQUENCE (5'-3')
PADI1 FW	TCCAGAGACCCTGAAGCTGT
PADI1 RV	GTGCAGCTGTCCCTGAAGAT
PADI2 FW	ACCTCCTCAGCCTCCCC
PADI2 RV	CCTACCTCTGGACCGATGTC
PADI3 FW	GCGTCCCATAGACCTCAAAC
PADI3 RV	CAGAGAATCGTGCGTGTGTC
PADI4 FW	CCTGTGGATTTCTTCTTGGC
PADI4 RV	GGGCACCTTGACTCAGCTT
PADI6 FW	CAAGGTATAGGCGTGCTGGT
PADI6 RV	TCCTCCATACCTCCAAGGAA
IE1 FW	TCAGTGCTCCCTGATGAGA
IE1 RV	GATCAATGTGCGTGAGCACC

Supplementary Table 2. Primers used to prepare the *PADI2* and *PADI4* promoter reporter plasmids

NAME	SEQUENCE (5'-3')	POSITION
PADI4 Promoter FW	5'-TAACTCGAGGGCTCTATGAAGGCAGGA-3'	-1977/-1960
PADI2 Promoter FW	5'-TATCTCGAGCCCACACTGATTGTCATC-3'	-868/-850
PAD4 Promoter RV	5'- TAGAAGCTTCGTCGGGCTAGCTCGTC-3'	+27/+10
PAD2 Promoter RV	5'-TATAAGCTTCCTCCCCGCCGCAGTGC-3'	+63/+80

Position number +1 corresponding to the first nucleotide of the transcription initiation site.

Supplementary Table 3. CRISPR Guide RNAs

GENE OF INTEREST	gRNA TARGET SEQUENCE
PADI1 #1	gCATCAGTGTCTAGCGGCCAA
PADI2 #1	gACCTCTGGACCGATGTCTAC

PADI3 #1	gTTACCCATAAATGTCCACGA
PADI4 #1	gCAATATCCACGACCACCCCT
PADI6 #1	gCAGGTGGATGATACTCTGGA

Supplementary Table 4. Primers for TIDE analysis

GENE OF INTEREST	SEQUENCE (5'-3')
PADI1 FW	GGGGGTGTCCAAGGGTTTAC
PADI1 RV	TGGAGGTTTGCGTGTTTTGG
PADI2 FW	GATGACTTCGCCACGACCT
PADI2 RV	CGGAGGGTTACGGGTGAAAG
PADI3 FW	CACAGCTAAGTCCAACACCAG
PADI3 RV	CTAGGCTCATAGGCTCAGGATG
PADI4 FW	AGTAAGCACTGGCTGCTGTTT
PADI4 RV	ATCACGAGCTCTTCCACAGG
PADI6 FW	CCCCTTCTTGGGAATTGGGTG
PADI6 RV	GCAAAACAACGTGTTTCCCAG

PUBLICATIONS

viruses

MDPI

Communication

The Viral Tegument Protein pp65 Impairs Transcriptional Upregulation of IL-1β by Human Cytomegalovirus through Inhibition of NF-kB Activity

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Abstract: Interleukin-1β (IL-1β) is a key effector of the inflammasome complex in response to pathogens and danger signals. Although it is well known that assembly of the inflammasome triggers proteolytic cleavage of the biologically inactive precursor pro-IL-1β into its mature secreted form, the mechanism by which human cytomegalovirus (HCMV) regulates IL-1β production via the inflammasome is still poorly understood. Here, we show that the infection of human foreskin fibroblasts (HFFs) with a mutant HCMV lacking the tegument protein pp65 (v65Stop) results in higher expression levels of mature IL-1β compared to its wild-type counterpart, suggesting that pp65 mediates HCMV immune evasion through downmodulation of IL-1β. Furthermore, we show that enhanced IL-1β production by the v65Stop mutant is due in part to induction of DNA binding and the transcriptional activity of NF-κB. Lastly, we demonstrate that HCMV infection of HFFs triggers a non-canonical IL-1β activation pathway where caspase-8 promotes IL-1β maturation independently of caspase-1. Altogether, our findings provide novel mechanistic insights into the interplay between HCMV and the inflammasome system and raise the possibility of targeting pp65 to treat HCMV infection.

Keywords: human cytomegalovirus (HCMV); pp65; inflammasome; interleukin-1β (IL-1β); caspase-8

1. Introduction

Human cytomegalovirus (HCMV) is a ubiquitous opportunistic β-herpesvirus that is found in 50–90% of the population worldwide. Despite the occurrence of periodic reactivation and subsequent virus-shedding episodes, HCMV infection is asymptomatic in immunocompetent hosts. However, viral reactivation in immunocompromised hosts or infection of immunologically naïve fetuses in utero can lead to serious or life-threatening diseases, such as retinitis, deafness, mental retardation, malformations, and abortion [1,2].

Innate immunity is the first line of defense against HCMV, allowing the host to rapidly mount an antiviral response upon viral infection [3,4]. This is particularly prominent during the perinatal period when the immune system is still immature [3]. This innate response is mediated by type I and

III interferons (IFNs) and inflammatory cytokines, such as interleukin-1β, which very rapidly create an antiviral state in the host, thereby triggering the inflammatory response [5].

An important route of innate immunity relies on the inflammasome, a multimeric protein complex that is commonly formed by sensor proteins that are known as pattern recognition receptors (PRRs), which typically sense pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), resulting in the production of proinflammatory cytokines. In this complex, the adaptor molecule that is known as apoptosis-associated speck-like proteins containing a C-terminal caspase recruitment domain (ASC or Pycard) bridges cytosolic PRRs and pro-caspase-1, with the latter being converted from an inactive zymogen into an active protease, which then catalyzes the maturation of the inflammatory cytokines interleukin-1β (IL-1β) and interleukin 18 (IL-18).

Although a plethora of stimuli, such as nucleic acids, toxins, and metabolic products [6], trigger inflammasome assembly, the mechanism of inflammasome activation and inhibition in response to viral infections still remains partly understood. Most of what we know about inflammasome modulation during cytomegalovirus (CMV) infection derives from experiments with mouse CMV (MCMV) [7,8], even though there have been few reports showing inflammasome activation in HCMV-infected monocytes or macrophages [7,8]. Among HCMV proteins, the tegument protein pp65 (pUL83) seems to play a major role in immunomodulation and immune evasion [9–13]. Downmodulation of caspase-1 and IL-1β activation has indeed been observed following the interaction of pp65 with the DNA sensor that is absent in melanoma 2 (AIM2) [12]. Yet, Li et al. [14] showed that pp65 can bind the pyrin domain of all nuclear pyrin and HIN domain (PYHIN) proteins (i.e., IFI16, IFIX, and MNDA), however not the AIM2-PY domain. Notably, a pp65 deletion mutant of HCMV was reportedly unable to induce inflammasome activity to levels that were comparable to that of wild-type HCMV, as judged by caspase-1 cleavage. Moreover, they found no changes in caspase-1 cleavage during HCMV infection, which led them to hypothesize that the canonical inflammasome assembly pathway does not play any role during enhanced IL-1β production upon HCMV infection [14]. Thus, despite this large body of literature, there is still much controversy surrounding the role of pp65 in the modulation of HCMV evasion mechanisms.

The aim of our study was to shed light on the molecular mechanisms of HCMV-mediated upregulation and activation of the IL-1β gene, focusing on the role of pp65. Here, we demonstrate that: (i) HCMV infection induces IL-1β expression and release, and that this effect is significantly strengthened upon infection with an HCMV pp65 mutant, which is unable to express *UL83*-encoded pp65 (v65Stop); (ii) NF-κB is a relevant transcription factor that is involved in the IL-1β promoter activation; and finally, (iii) HCMV primes IL-1β expression in a caspase-8 dependent manner.

Altogether, our results confirm and expand the key role of the tegument protein pp65 in the modulation of innate immunity.

2. Materials and Methods

2.1. Cells and Viruses

Primary human foreskin fibroblasts (HFFs, ATCC SCRC-1041™) and human embryo kidney 293 cells (HEK 293) (Microbix Biosystems Inc., Mississauga, ON, Canada) were cultured in Dulbecco's modified Eagle's medium (DMEM), which was supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich, Milan, Italy), as we previously described [15]. The HCMVs that were used in this study were all bacterial artificial chromosome (BAC) clones. The clones of the endotheliotropic HCMV strain TB40/E wild-type and a mutant virus that is unable to express *UL83*-encoded pp65 (v65Stop) have been described previously [16]. The viruses were propagated on HFFs and were titrated by standard plaque assay [15]. HCMV infections were all performed at a multiplicity of infection (MOI) of 1. UV-inactivated HCMV were prepared using a double pulse of UV-B light (1.2 J/cm^2). The UV-inactivated HCMV did not replicate or produce detectable levels of immediate-early (IE) gene products.

2.2. Recombinant Adenoviral Vectors

The adenovirus-derived vectors (AdV) expressing pp65 were generated by means of a replacement strategy using recombineering methods as described previously [11].

2.3. Luciferase Assay

IL-1β promoter sequence was cloned into a luciferase pGL3-promoter vector (Promega, Madison, WI, USA). The IL-1β promoter sequences were amplified using specific sets of primers: IL-1β Fw KpnI 5′-CGGGTACCCAGCACCCAAGGTAGAGACC-3′; IL-1β Rev XhoI 5′-CGCTCGAGTGTTGGATCTTGAGGCCTAA-3′; mut-IL1-β Fw KpnI 5′-CGGGTACCTAATGTGGACATCAACTGCA-3′. The luciferase reporter constructs containing the wild-type (pIL1-β-WT) and deletion mutant (pIL1-β-NF-κB-KO) IL-1β promoter fragments and the pRL-SV40 (Promega, Madison, WI, United States) plasmid were transiently electroporated into HFFs as previously described [17]. Twenty-four hours later, they were infected with wild-type or v65Stop. Following a further 24 h post infection (hpi), firefly and Renilla luciferase activities were measured, as previously described [18], using the Dual-Luciferase reporter assay system kit (Promega, Madison, WI, USA) and a Victor X3 Multilabel Plate Reader (Perkin Elmer, Waltham, MA, USA). Firefly luciferase activity from the luciferase reporter vector was normalized to the Renilla luciferase activity from the pRL-SV40 vector. The data report the ratio of relative light units (RLU) that were measured for firefly luciferase activity to the RLU that were measured for Renilla luciferase activity.

2.4. RNA Isolation and Semiquantitative RT-qPCR

Total RNA was extracted using the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) and 1 μg was retrotranscribed using the Revert-Aid H-Minus FirstStrand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol. The comparison of mRNA expression between the samples (i.e., infected versus untreated) was performed by SYBR green-based RT-qPCR using Mx3000P apparatus (Stratagene, San Diego, CA, USA) using the following primers: IL-1β Fw TCCCCAGCCCTTTTGTTGA, IL-1β Rw TTAGAACCAAATGTGGCCGTG; the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Fw AGTGGGTGTCGCTGTTGAAGT, GAPDH Rw AACGTGTCAGTGGTGGACCTG.

2.5. Immunofluorescence Microscopy

Indirect immunofluorescence analysis was performed as previously described [19]. The following primary antibodies were used: rabbit polyclonal anti-IEA (Santo Landolfo, University of Turin, Italy), and mouse monoclonal anti-NF-κB p65 (Santa Cruz Biotechnology, Dallas, TX, USA). Signals were detected using goat anti-rabbit or goat anti-mouse conjugated secondary antibodies (Thermo Fisher Scientific, Waltham, MA, USA). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The samples were observed using a fluorescence microscope (Olympus IX70, Olympus Italia, Segrate, Italy) that was equipped with cellSens Standard-Microscopy Imaging Software. ImageJ software was used for image processing.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

Cell-free supernatants were harvested and IL-1β production was measured by DuoSet ELISA assay according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). All absorbance readings were measured at 450 nm using an ELISA Plate Reader (DAS, Palombara Sabina, Italy).

2.7. Non-Radioactive Universal EZ-TFA Transcription Factor Assay

The DNA binding activity of p65/RelA was measured using a Universal EZ-TFA transcription factor assay colorimetric kit (Upstate Biotechnology Inc., Lake Placid, NY, USA) according to the

manufacturer's protocol. In brief, a double-stranded biotinylated oligonucleotide containing the consensus sequence for p65 was used as a capture probe; it was mixed with nuclear extracts and was added directly into the streptavidin-coated plate. An unlabeled oligonucleotide containing the identical consensus sequence as the capture probe was used as a competitor. Any inactive, unbound material was washed away and the bound p65 was detected with a specific primary antibody. A horseradish peroxidase (HRP)-conjugated secondary antibody was then used for detection, and p65 specific binding was quantified at 450 nm using a microplate reader. The p65 probe sequences were: sense (biotin): 5′-ATGACATAGGAAAACTGAAAGGGAGAAGTGAAAGTGGGAAATTCCTCTG-3′; antisense: 5′-CAGAGGAATTTCCCACTTTCACTTCTCCCTTTCAGTTTTCCTATGTCAT-3′. Unlabeled oligonucleotide was added as the competitor DNA.

2.8. In Vitro Analysis of Caspase-1 and Caspse-8 Activity

Caspase-1 and caspase-8 protease activity was measured by evaluating the extent of cleavage of a fluorometric peptide substrate using SensoLyte AFC Caspase Sampler Kit Fluorimetric (Anaspec, CA, USA). The experiments were performed according to the manufacturer's instructions. After an hour of incubation at 25 °C, fluorescence was measured at an excitation wavelength of 405 nm and an emission wave length of 500 nm using the Victor X3 Multilabel Plate Reader (Perkin Elmer, Waltham, MA, USA). Protease activity was expressed as the fold induction of HCMV-infected vs. mock-infected cells.

2.9. Inhibition of Caspase-1 and Caspase-8 Expression

HFFs were transiently transfected with a MicroPorator (Digital Bio Pharm, London, Great Britain) according to the manufacturer's instructions (1200 V, 30 ms pulse width, one impulse) with a pool of small interfering RNAs (Qiagen, Hilden, Germany) targeting caspase-1 (siCASP1, FlexiTube siRNAs cat. No.: SI0266244304132170, SI0266193204263189, SI0265459604287626, SI0494828604357696), caspase-8 (siCASP8, FlexiTube siRNAs cat. No.: SI0266245704359754, SI0266194604210101, SI0029959304164797, SI0494831404131687), or control siRNA (siCTRL, 1027292) as negative control.

2.10. Statistical Analysis

All statistical tests were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA). The data were presented as means ± standard deviations (SD). Statistical significance was determined by using two-tailed Student's *t*-tests; one-way or two-way analysis of variance (ANOVA) with Bonferroni's post-tests, as appropriate. Differences were considered statistically significant for $p < 0.05$ ($p < 0.05$ *; $p < 0.01$ **; $p < 0.001$ ***).

3. Results and Discussion

3.1. IL-1β Induction upon HCMV Infection is Inhibited by the HCMV Tegument Protein pp65 in an NF-κB Dependent Manner

HCMV infection induced the production of IL-1β in different cell types [20–22]. Moreover, we and others have identified the HCMV tegument protein pp65 [9–12] as one of the key mediators of HCMV evasion from the innate immune response. Thus, we sought to determine whether the immunosuppressive function of pp65 could be mediated by the inflammasome system. To this end, HFFs were first mock-infected or infected with wild-type HCMV or v65Stop HCMV, a mutant that is unable to express *UL83*-encoded pp65 [11,16], at an MOI of 1, and total RNA was analyzed by RT-qPCR at 6 and 24 hpi. HCMV-IEA mRNA expression was employed as a positive control for viral infection (data not shown). As shown in Figure 1A, IL-1β mRNA levels that were observed at 24 hpi in v65Stop-infected HFFs were approximately 1.7-fold higher than those that were observed in cells that were infected with wild-type HCMV, indicating that pp65 may negatively regulate *IL-1β* gene expression.

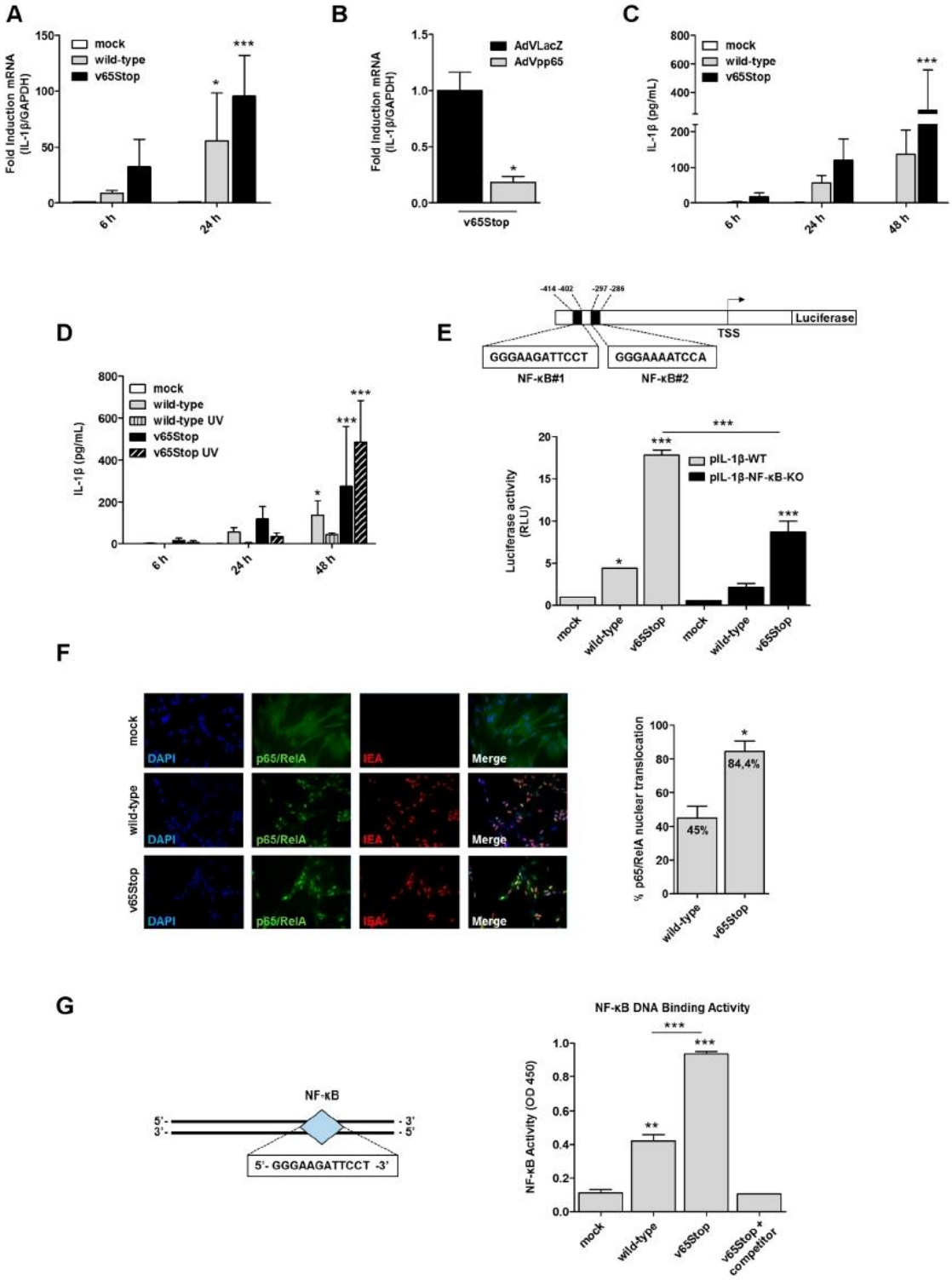

Figure 1. HCMV (human cytomegalovirus) pp65 inhibits IL-1β (interleukin-1β) response following HCMV infection of HFFs (human foreskin fibroblasts) through NF-κB. (**A**) HFFs were infected at an MOI of 1 with wild-type or v65Stop HCMV and were analyzed by RT-qPCR. IL-1β mRNA expression levels following HCMV vs. mock infection were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and are shown as mean fold changes ± SD (*, $p < 0.05$; ***, $p < 0.001$; two-way ANOVA followed by Bonferroni's post-tests). (**B**) HFFs were transduced with AdVLacZ (black bar) or AdVpp65 (grey bar) at an MOI of 50. Subsequently, cells were infected with v65Stop HCMV (MOI of 1). At 15 hpi,

IL-1β mRNA expression was normalized to that of GAPDH and is shown as a mean ± SD fold change (*, $p < 0.05$; unpaired *t*-test). (**C**) HFFs were infected with wild-type or v65Stop HCMV at an MOI of 1. Supernatants were collected at 6, 24, and 48 hpi and were assessed by ELISA for IL-1β production. Results are shown as a mean ± SD fold change (***, $p < 0.001$; two-way ANOVA followed by Bonferroni's post-tests). (**D**) HFFs were infected with wild-type, wild-type UV, v65Stop, or v65Stop UV at an MOI of 1. Supernatants were collected at 6, 24, and 48 hpi and were assessed by ELISA for IL-1β production. Results are shown as the mean ± SD fold change (*, $p < 0.05$; ***, $p < 0.001$; two-way ANOVA followed by Bonferroni's post-tests). (**E**) Schematic representation of the IL-1β luciferase promoter plasmid with the sequences containing the two putative NF-κB binding sites indicated as NF-κB#1 and NF-κB#2 (left panel). HFFs were transiently electroporated with luciferase plasmids encoding the wild-type (pIL-1β-WT) or deletion mutant (pIL-1β-NF-κB-KO) IL-1β promoter fragments, and pRL-SV40. Twenty-four hours later, the cells were mock-infected or infected with wild-type or v65Stop HCMV at an MOI of 1. At 24 hpi, firefly and Renilla luciferase activities were measured. The luciferase activity in whole-cell lysates was normalized to Renilla luciferase activity and is expressed as relative light units (RLU) (right panel). Results are shown as the mean ± SD fold change (*, $p < 0.05$; ***, $p < 0.001$; one-way ANOVA followed by Bonferroni's post-tests). (**F**) HFFs mock-infected or infected with wild-type or v65Stop HCMV at an MOI of 1 were fixed at 24 hpi and were subjected to immunofluorescence analysis. HCMV-IEA (red) and p65/RelA (green) were visualized using primary antibodies, followed by secondary antibody staining, in the presence of 10% HCMV-negative human serum. Nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole) (blue). The graph (right panel) shows the levels of nuclear NF-κB in infected cells. The data represent the mean fold changes (*, $p < 0.05$; unpaired *t*-test). (**G**) Schematic representation of the probe containing the NF-κB#1 binding site (left panel). HFFs were left untreated (mock) or infected with wild-type or v65Stop (MOI of 1). At 24 hpi, the cells were lysed and the nuclear fraction was analyzed for NF-κB binding activity using the Universal EZ-TFA transcription factor assay colorimetric kit (right panel). The data show the means ± SD (**, $p < 0.01$; ***, $p < 0.001$; one-way ANOVA followed by Bonferroni's post-test).

To test whether ectopic expression of pp65 would downregulate IL-1β gene expression, we either infected HFFs constitutively expressing pp65 protein (AdVpp65) or not (AdVLacZ) with v65Stop HCMV for 15 h. Consistent with our previous results, RT-qPCR analysis revealed that IL-1β mRNA expression levels were reduced by approximately 80% in AdVpp65-infected cells compared to the control (Figure 1B). Thus, these findings suggest a model whereby pp65 plays an essential role in HCMV escape from the host immune response through the downmodulation of IL-1β gene expression.

Next, we asked whether enhanced levels of IL-1β mRNA in v65Stop-infected HFFs would correlate with increased production of biologically active IL-1β protein. For this purpose, supernatants from HFFs that were infected with wild-type or v65Stop viruses were harvested at 6, 24, and 48 hpi and were assessed by ELISA for IL-1β production. As shown in Figure 1C, and consistent with the results obtained with RT-qPCR, the levels of IL-1β that were secreted at 24 and 48 hpi are considerably higher in v65Stop HCMV-infected cells than those that were observed in wild-type HCMV-infected ones, indicating that in the presence of HCMV pp65, the signaling pathway leading to IL1-β production is impaired (Figure 1C). After viral envelope fusion, the virion-associated pp65 is released into the cytoplasm and subsequently translocates to the cell nucleus. To determine whether pp65 protein delivery is sufficient for downmodulation of IL-1β production or whether viral gene expression is required, we employed the UV-inactivated viruses wild-type and v65Stop (named wild-type UV and v65Stop UV). In detail, supernatants from HFFs that were infected with wild-type, wild-type UV, v65Stop, and v65Stop UV viruses were harvested at different times post infection (6–48 hpi) and were assessed by ELISA for IL-1β production. As shown in Figure 1D, the levels of secreted IL-1β are significantly lower in the wild-type and wild-type UV -infected cells than those that were observed in v65Stop and v65Stop UV, and this effect is more appreciable at 48 hpi. Overall, these results support our hypothesis that virion-associated pp65 released in the cytoplasm in the early stage of infection contributes significantly to the modulation of IL-1β production (Figure 1D).

Since HCMV infection results in NF-κB dysregulation [9,23–25], and the IL-1β promoter contains two putative binding sites for the NF-κB transcription factors, located at positions −412 to −402 (5′-GGGAAGATTCCT-3′) and -297 to -286 (5′-GGGAAAATCCA-3′) (Figure 1E) [26], we sought to determine whether downmodulation of IL-1β gene expression occurred at the transcriptional level due to inhibition of NF-κB activity. For this purpose, the luciferase reporter constructs containing the wild-type (pIL1-β-WT) and deletion mutant (pIL1-β-NF-κB-KO) IL-1β promoter fragments were transiently transfected into HFFs. Twenty-four hours later, the cells were left uninfected or infected with wild-type or v65Stop HCMV. Luciferase activity was then assessed following an additional 24 h of incubation. As shown in Figure 1E, v65Stop HCMV-infected HFFs display a 4-fold induction of pIL-1β-WT luciferase activity compared to that of the cells that were infected with wild-type HCMV. By contrast, the induction of pIL-1β-NF-κB-KO luciferase activity was decreased by approximately 50% in the cells that were infected with either v65Stop or wild-type HCMV. Thus, deletion of NF-κB binding sites downregulates IL-1β promoter activity following HCMV infection, suggesting that the downregulation of IL-1β gene expression by v65Stop HCMV occurs at the transcriptional level and that it is likely mediated by inhibition of NF-κB activity. However, the residual activity of IL-1β promoter observed in pIL-1β-NF-κB-KO suggests that other transcription factors may be involved in the regulation of IL-1β promoter activity.

NF-κB is a heterodimer consisting of a 50-kDa subunit (p50) and a 65-kDa subunit (p65/RelA). Under normal physiological conditions, NF-κB is sequestered in the cytosol by a family of IκB inhibitors in an inactive state [9,23,27]. To ascertain whether HCMV infection promotes RelA translocation to the nucleus and whether this effect is opposed by pp65, HFFs were infected with wild-type or v65Stop for 24 h and were analyzed by indirect immunofluorescence using anti-RelA antibodies. As shown in Figure 1F, 45% of wild-type infected cells display RelA nuclear translocation. By contrast, and consistent with the results reported by Browne et al. [9], nuclear translocation of RelA was more pronounced in cells that were infected with v65Stop (84.4% of infected cells), indicating a stronger activation of NF-κB in the absence of HCMV pp65.

Upon its translocation to the nucleus, RelA binds to specific response elements in the promoter regions of responsive genes. Having observed that pp65 impairs the nuclear translocation of RelA, we next sought to determine whether pp65 could inhibit DNA binding of RelA to its consensus site, thereby preventing its transcriptional activity. To this end, we took advantage of the Universal EZ-TFA transcription factor colorimetric assay, which combines the DNA-binding principle of the electrophoretic mobility shift assay with the 96-well format of an ELISA assay [28,29]. In this context, nuclear extracts from v65Stop HCMV-infected cells exhibited an approximately 50% increase in RelA DNA-binding activity compared to nuclear extracts from HFFs that were infected with wild-type HCMV at 24 hpi (OD 450 of p65—DNA binding, wild-type vs. v65Stop HCMV infected cells: 0.4 vs. 0.9) (Figure 1G). Altogether, these results demonstrate that pp65 inhibits IL-1β gene expression at the level of transcription by reducing binding of RelA to at least one of the two κB sites within the IL-1β promoter.

3.2. HCMV Primes IL-1β Activation in a Caspase-8 Dependent Manner

The maturation step of bioactive IL-1β has long been thought to be catalyzed solely by the inflammatory cysteine protease caspase-1, also known as ICE (IL-1β-converting enzyme) [30]. However, recent evidence indicates alternative pathways of IL-1β release independent of inflammasome-induced caspase-1 activation. In this regard, caspase-8 has recently been identified as an alternative protease that can process IL-1β independently of the inflammasome leading to the secretion of the active form [30–32]. Furthermore, studies in HEK293T cells overexpressing both caspase-8 and pro-IL-1β have shown that caspase-8 can directly cleave pro-IL-1β in response to stimulation by TLR3 or TLR4. Furthermore, stimulation of dectin-1, a macrophage-specific C-type lectin receptor, has been shown to lead to caspase-8 activation and caspase-1-independent maturation of pro-IL-1β [32].

Based on these findings, we compared the activity of caspase-1 and caspase-8 at different time points after infection with wild-type or v65Stop HCMV-infected HFFs. In agreement with Li et al. [14],

we failed to detect changes in caspase-1 activity under any conditions, suggesting that caspase-1 activity does not play a role during inflammasome assembly following HCMV infection (Figure 2A). By contrast, we observed a significant activation of caspase-8 activity in HFFs that were infected with either HCMV strains compared to the mock (Figure 2A). Interestingly, there was no significant difference in the ability to activate caspase-8 between the two virus strains, suggesting that HCMV pp65 decreases IL-1β at the transcriptional level, however that it does not interfere with caspase activation.

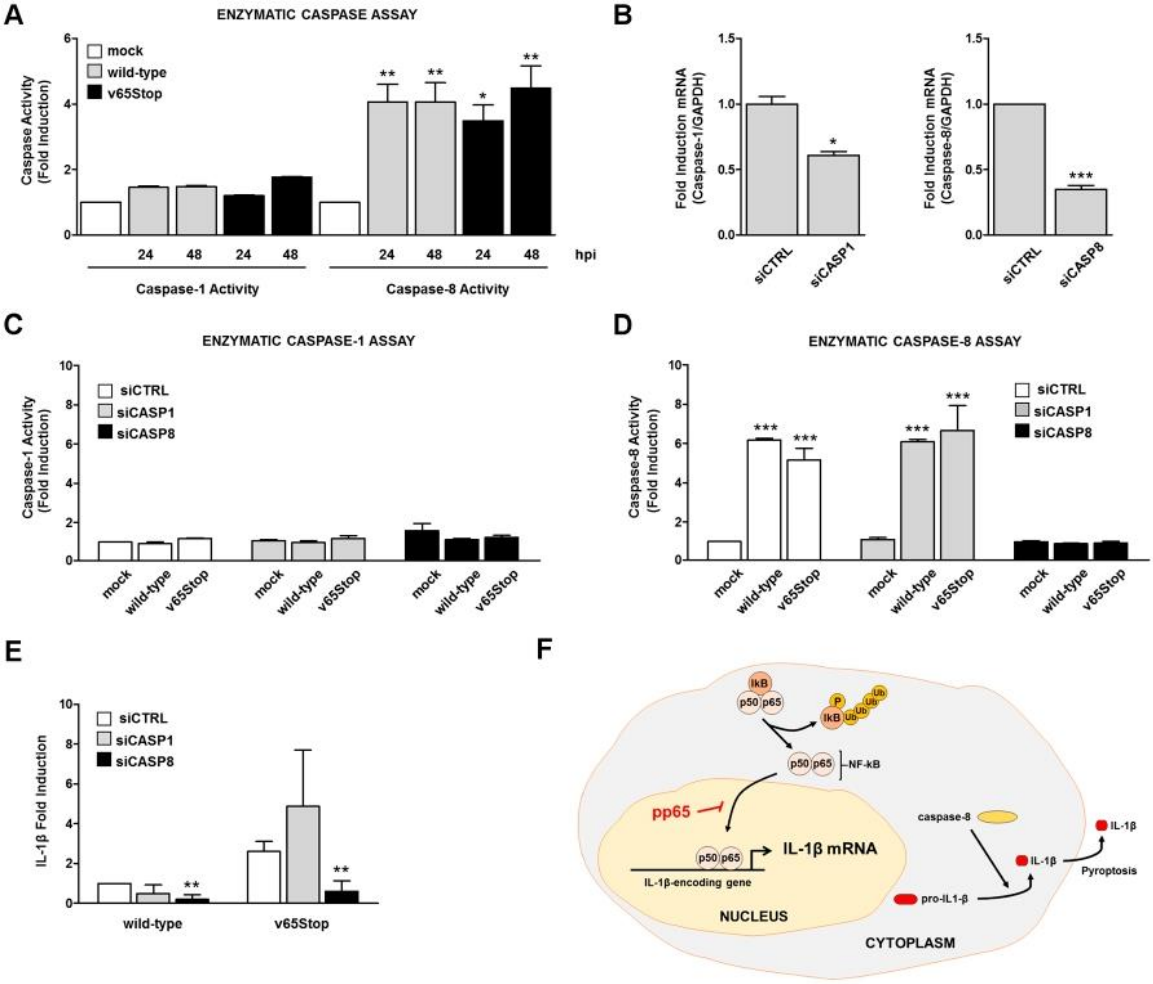

Figure 2. HCMV primes IL-1β activation in a caspase-8 dependent manner. (**A**) HFFs were infected with wild-type or v65Stop HCMV at an MOI of 1 for 24 and 48 h and were processed by fluorimetric assay for caspase-1 and caspase-8 activation. Fold changes were calculated after normalization of HCMV vs. mock-infected cells. Data are shown as mean ± SD (*, $p < 0.05$; **, $p < 0.01$; one-way ANOVA followed by Bonferroni's post-tests). (**B**–**D**) HFFs were electroporated with pools of siRNA targeting caspase-1 (siCASP1), caspase-8 (siCASP8), or scrambled control siRNA (siCTRL). (**B**) The efficiency of caspase-1 and caspase-8 depletion was assayed by RT-qPCR (the data are shown as mean fold changes plus SD; *, $p < 0.05$; ***, $p < 0.001$; by unpaired *t*-test). (**C**,**D**) siCTRL, siCASP1, and siCASP8 HFFs were infected with wild-type or v65Stop HCMV at an MOI of 1 and were examined at 24 hpi by fluorimetric assay for caspase-1 (**C**) and caspase-8 (**D**) activation. Results are shown as the mean ± SD fold change (***, $p < 0.001$ two-way ANOVA followed by Bonferroni's post-tests). (**E**) Cells were infected as described in (**C**,**D**). Supernatants were collected at 24 hpi and were analyzed by IL-1β ELISA. Results are shown as the mean ± SD fold change over wild-type siCTRL set as 1.0. A statistically significant difference compared to siCTRL is indicated by asterisks (**, $p < 0.05$; unpaired *t*-test). (**F**) Model depicting the proposed functional role of pp65 modulation of IL-1β activity during HCMV infection.

It is relevant to know that HCMV *UL36* encodes for a cell death suppressor, named pUL36 or vICA, which binds to the pro-domain of caspase-8 and prevents its activation, thereby blocking the Fas-mediated apoptosis-signaling pathway [33]. In addition, Skaletskaya and colleagues [33] showed that a strain-specific single point-mutation observed in AD169*var*ATCC (Cys ^{131} → Arg ^{131}) affects the binding of vICA to pro-caspase-8 and consequently abrogates its antiapoptotic activity.

In order to assess whether the two HCMV strains that were used in our experiments carried an intact *UL36* gene, *UL36* regions were sequenced and were compared with the published sequences from TB40/E, AD169*var*ATCC, and the Towne strain, which was shown to induce caspase-8 activity in HFFs at later time points after infection [34]. An alignment of the *UL36* sequences from the different HCMV strains confirmed that our HCMV strains do not carry the mutation found to render pUL36 inactive, suggesting that at earlier time points of the infection, pUL36 could still be able to inhibit caspase-8 activity, however that a residual amount of pro-caspase-8 gets activated and cleaves IL-1β (data not shown).

To strengthen the specificity of these results, we performed ablation experiments using a mixture of specific siRNAs targeting caspase-1 (siCASP1), caspase-8 (siCASP8), or scrambled control siRNA (siCTRL). The efficacy of silencing was assessed by RT-qPCR (Figure 2B) and by caspase activity (Figure 2C,D). As shown in Figure 2C, no caspase-1 activity was detected under any conditions. By contrast, siCTRL- and siCASP1-treated cells showed increased caspase-8 activity in response to HCMV infection (Figure 2D).

Next, to assess more directly the involvement of caspase-1 and/or caspase-8 in IL-1β maturation, we performed an ELISA specific for IL-1β using supernatants that were obtained from HFFs that were transiently depleted of caspase-1 or caspase-8, respectively, and were then infected with wild-type or v65Stop HCMV for 24 h. A significant decrease in IL-1β production was observed in cells that were depleted for caspase-8, however not caspase-1, which were then infected with wild-type or v65Stop HCMV (Figure 2E), indicating that caspase-8 is necessary for maturation of IL-1β during HCMV infection. In agreement with the enzymatic activity assay, a residual induction of IL-1β release was still present in HCMV-infected caspase-8-deficient HFFs compared to siCTRL- or siCASP1-treated cells, implying that other factors may contribute to IL-1β production. Altogether, these results indicate that caspase-8 is essential to mount an abundant IL-1β maturation in response to HCMV infection.

4. Conclusions

Our findings add novel functional and mechanistic insights into the role that is played by the HCMV tegument protein pp65 in HCMV immune evasion. In this regard, we show that the production of mature IL-1β is significantly increased upon infection with an HCMV mutant that is unable to express pp65. Furthermore, the observation that the infection of HFFs with the v65Stop HCMV mutant leads to the induction of both NF-κB DNA binding and transcriptional activities at promoter regions of IL-1β, thereby enhancing the production of IL-1β, strongly argues in favor of an inhibitory role that is played by HCMV pp65 during IL-1β production. Equally important, we show for the first time that a non-canonical pathway has evolved in response to HCMV infection, leading to inflammasome-independent maturation of IL-1β via caspase-8 activation (Figure 2F).

Overall, our studies further confirm and expand the prominent role of the tegument protein pp65 in the modulation of the innate immune response, a function that could be exploited therapeutically.

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Conflicts of Interest: The authors declare no conflict of interest.

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Review

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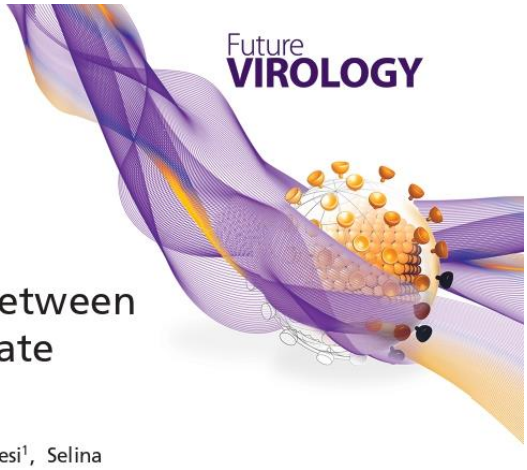

Catch me if you can: the arms race between human cytomegalovirus and the innate immune system

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Human cytomegalovirus (HCMV), a common opportunistic pathogen of significant clinical importance, targets immunocompromised individuals of the human population worldwide. The absence of a licensed vaccine and the low efficacy of currently available drugs remain a barrier to combating the global infection. The HCMV's ability to modulate and escape innate immune responses remains a critical step in the ongoing search for potential drug targets. Here, we describe the complex interplay between HCMV and the host immune system, focusing on different evasion strategies that the virus has employed to subvert innate immune responses. We especially highlight the mechanisms and role of host antiviral restriction factors and provide insights into viral modulation of pro-inflammatory NF-κB and interferon signaling pathways.

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Background

Human cytomegalovirus (HCMV), a prototypic β-herpesvirus, is a common host-restricted opportunistic pathogen that contains the largest genome among all known human viruses, and which is capable of successful establishment of a lifelong persistence with spontaneous reactivation periods within the infected hosts [1,2]. An important clinical pathogen, HCMV is widely spread in humans all around the globe, with seroprevalence ranging between 40 and 100% of the susceptible population and likely to be highest in countries with lower socioeconomic conditions. Generally, it causes mild or asymptomatic infection in the immunocompetent, but it often leads to severe complications and even mortality in immunocompromised hosts, such as cancer patients, organ transplant recipients under immunosuppressors or AIDS patients [3,4]. Neonates with immature immune systems are also at high potential risk of HCMV congenital infection, which often leads to severe birth defects and permanent neurological morbidities, such as deafness, blindness and long-term intellectual disability in infected newborns [5–8]. Furthermore, HCMV may contribute to immunosenescence in the elderly [9,10] and to a number of autoimmune [11–14], inflammatory and vascular diseases [15–19], as well as some cancers [20–24].

However, despite its clinical importance, there are currently no available vaccines to prevent the spread of infection and only a few licensed antiviral drugs, which are limited by their low efficacy, high hematopoietic toxicity and poor bioavailability [25–27]. Furthermore, while these drugs target the HCMV during its lytic replication cycle, they remain useless against the latent infection. On top of it all, the emergence of antiviral resistance among HCMV strains has recently become a highly concerning and deeply threatening issue in clinical management of immunocompromised patients, widely reported in all the risk groups [28–31].

To successfully establish a latent infection, HCMV has adopted a series of elaborate approaches to suppress host immune responses, allowing it to achieve wide dissemination within the infected host [32]. As a virus with an enormously large genome, encoding over 200 open reading frames (ORFs), HCMV potentially employs hundreds of proteins with modulatory functions to enable viral replication and immune evasion, targeting both innate and adaptive immune responses via distinct mechanisms and biochemical pathways.

Future Medicine

In spite of multiple evasion strategies of HCMV, the host immune system is still capable of counteracting the infection by building up a robust immune response in wide frontiers, for instance, by involving various DNA sensors and host restriction factors (RFs). Indeed, there is broad evidence that primary HCMV infection in immunocompetent individuals are generally asymptomatic, while immunocompromised individuals experience the full and severe blast of HCMV disease.

Here, we discuss the complex 'arms race' between HCMV and the host, reflected in the multiple evasion strategies HCMV has evolved to successfully escape the innate immunity of the host. We particularly highlight the mechanisms and role of various RFs involved in the antiviral response, along with the newest insights into viral modulation of pro-inflammatory NF-κB and interferon (IFN) signaling pathways.

Considering the importance of predicting HCMV infection outcomes, it is key to understand the process of HCMV immunomodulation in order to expand our knowledge of viral pathogenesis, which may contribute to the development of effective HCMV vaccines and/or therapeutic interventions.

Innate immunity versus HCMV infection: a brief overview

Innate immunity represents the antiviral frontline and has traditionally been considered a major protective mechanism in effectively combating the replication of viruses, including HCMV. In addition to robust production of interferons, natural killer (NK) and antigen-presenting cells (APCs) are also enrolled and activated. Recently, a prominent role of intrinsic immune mechanisms has been discovered, as a part of the antiviral frontline barrier mediated by a number of cellular proteins, namely RFs. Unlike other innate immunity players, RFs are constitutively expressed and active, also prior to pathogen's entry into a cell. Remarkably, a close interplay occurs between innate and intrinsic immunity, as demonstrated by interferon upregulation of several RFs, increasing their antiviral activity [33].

HCMV components rapidly activate myeloid cells, such as monocytes, macrophages and myeloid dendritic cells, emphasizing the significance of early virus–host interactions and serving as a trigger in the activation of immediate immune responses [34].

In particular, NK cells are widely acknowledged as major host defenders in the fight against HCMV. They detect HCMV-infected cells, using a plethora of stimulatory and inhibitory receptors on their cell surface, responsible for NKs activation, proliferation, as well as their effector functions. In its turn, HCMV employs a repertoire of immunoevasive strategies directed against NK activity; therefore, confirming the crucial role of NKs in promotion of the innate resistance to HCMV [35].

First, HCMV engages inhibitory receptors using numerous multifunctional immunoevasive proteins against several and often unrelated targets. For example, the HCMV-encoded viral IL-10 ortholog (vIL-10), which modulates the early host immune system in favor of HCMV by decreasing the population size of NK effector cells, overall promotes viral persistence in the immunocompetent organism [36]. In addition, another HCMV evasion mechanism from NKs employs an active downregulation of NKG2D ligands by using several potent decoy molecules encoded by HCMV. It is well established that viral pUL40 interacts with HLA-E, upregulating its surface expression, and therefore, enabling its binding with the inhibitory receptor CD94/NKG2A. Another HCMV protein, pUL18, acts as an HLA-I homolog, interacting with the inhibitory receptor LIR-1. Likewise, pUL16 suppresses the expression of the ligands of the activating receptor NKG2D, targeting ULBP1, ULBP2 and MICB, whereas miR-UL112 inhibits ULBP2, while pUL142, US9, US18 and US20 target MICA and ULBP3. On top of it all, pUL141 inhibits CD112 and CD155 ligands of the activating CD226 and CD96 receptors, while pp65 mediates inhibition of NK cytolysis of HCMV-infected fibroblasts via NKp30 inhibition [37].

At last, HCMV encodes a range of Fc receptors with cell surface localization and concomitant incorporation into virions; thereby, interfering with NK-mediated cytotoxicity and complement attack [38].

Cellular immunity activation is then followed by humoral immune responses. In this context, antibody-mediated complement cytolysis represents an essential defense mechanism in which elimination of virus-infected cells may be accomplished. Conversely, HCMV have developed strategies to subvert complement activity. For example, HCMV incorporates two complement regulators, CD59 and CD55, into its viral particles [39]. Moreover, HCMV also increases the cell surface expression of CD46 and CD55; thereby, suppressing the accumulation of C3 convertases, which shield the cells from complement-mediated cytolysis [40]. Finally, a downregulation of complement receptors CD11b/CD18 (CR3) and CD11c/CD18 (CR4) has been previously observed in a monocytic THP-1 cells and macrophages upon infection, resulting in decreased phagocytosis by macrophages [41].

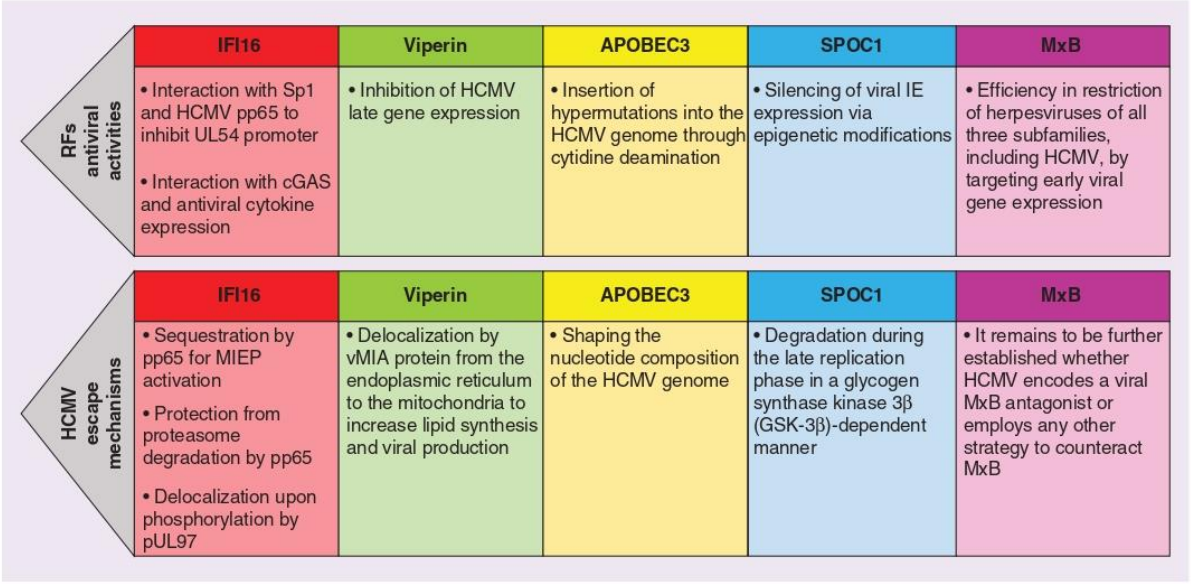

Figure 1. The best-characterized host restriction factors in the defense against human cytomegalovirus and viral countermechanisms.

HCMV restriction factors

It is well known that susceptibility to viral infection is partly determined by RFs. RFs represent a wide group of host proteins that 'restrict' viral replication by directly essential viral and/or cellular genes; thereby, providing a frontline defense against invaders. During the evolutionary 'arms race' for survival, viral proteins have successfully evolved to modulate or degrade RFs.

Early retroviral studies have identified two major host RFs: the apolipoprotein B editing catalytic subunit-like 3 (APOBEC3) proteins and tetherin [42–45]. A major research focus on inhibitory molecules and their restriction mechanisms in the following years has illuminated a significant number of newly discovered RFs, potentially able to counteract other viruses, including HCMV [46]. So far, several host proteins, including γ-interferon-inducible protein 16 (IFI16), viperin, nuclear domain 10 (ND10) complex, APOBEC3 cytidine deaminases, survival time-associated PHD protein in ovarian cancer 1 (SPOC1) and myxovirus resistance B (MxB) have been proposed to counteract HCMV infection by restricting viral replication. Interestingly, HCMV, in its turn, has evolved effective countermeasures to resist them (Figure 1). Below, we discuss the above mentioned RFs in detail, leaving out nuclear domain 10 even though it is a very important RF of HCMV, because this topic has been previously addressed in numerous works [47–54].

γ-Interferon-inducible protein 16 (IFI16)

IFI16 is a widely known key player in the intrinsic resistance to a variety of viruses. Over the last decade, IFI16 antiviral activity has been extensively studied and found possible restriction activity has been proposed in context of several viral infections, including HCMV [55–57]. Intriguingly, IFI16 demonstrates a controversial dual nature as a proviral agent upon early stages of HCMV infection, but acts as a repressor of viral gene transcription later on. In greater detail, early during infection, IFI16 is being recruited by viral pp65 to the viral major immediate-early promoter (MIEP), facilitating the upregulation of immediate-early (IE) protein expression, followed by a concomitant decrease of cytokine production, while at later time points, IFI16 is potentially protected by pp65 from proteasome degradation, which sustains its inhibitory activity at the *UL54* gene promoter [55,58]. In this context, the work by Gariano *et al.* [55] has also shown that the knockdown of IFI16 expression in human fibroblasts results in significantly increased HCMV replication. In agreement with these data, overexpression of IFI16 led to inhibition of viral growth. Potentially, IFI16 antiviral effectiveness depends on its ability to block Sp1-like transcription factors on the viral UL54 promoter [55].

However, later during infection, HCMV mediates IFI16 nucleus–cytoplasm translocation, thus subverting its antiviral activity. UL97, a viral-encoded protein kinase, acts as a key mediator of the IFI16 nuclear translocation: upon binding to viral UL97, IFI16 is phosphorylated, which drives its nuclear egression. Later on, IFI16 becomes entrapped into the virus assembly complex (vAC) assisted by the endosomal sorting complex required for transport (ESCRT). Eventually, IFI16 is hijacked and trapped in the newly formed virions [59]. Along with UL97, HCMV pp65, another co-partner in crime, has recently been reported to be involved in HCMV escape by interacting with IFI16, targeting early gene promoters, such as UL54 [60]. For now, the interaction between pp65 and IFI16 remains a matter of significant debate.

The most recent findings have shown that cellular DNA sensor cyclic GMP–AMP synthase (cGAS) represents another interaction partner of IFI16, although at first glance these proteins appear functionally different. IFI16–cGAS interaction occurs via pyrin domain [61], but while IFI16 activates cytokine production, for example IFN-β, only cGAS is able to activate the STING/TBK-1/IRF3 signaling pathway and apoptosis upon herpes simplex virus type I (HSV-1) and HCMV infections in an effective manner [61,62]. Since pp65 tegument protein interferes with every component of the STING/TBK-1/IRF3 pathway to evade the interferon response, this clearly highlights the significance of the interferon system in counteracting viral replication. Thus, it may be beneficial to further elucidate the mechanisms through which HCMV interferes with cGAS/STING/IRF3, potentially enabling the development of therapeutic interventions targeting multiple diseases and syndromes in which this pathway is altered.

Viperin

Viperin, an interferon-inducible multifunctional protein, is upregulated in several cell types by a variety of viral pathogens, including HCMV. It possesses a wide range of critical functions, from acting as an antiviral protein by modulating cell signaling to being a proviral factor, and has therefore recently received increased attention due to its paradoxical role in innate immunity (proviral versus antiviral). It has previously been shown that viperin acts as an antiviral protein in the late stages of HCMV infection, as confirmed by the low gene expression of *pp65*, *gB* and *pp28* genes in fibroblasts expressing viperin [63].

It has been well established that viperin is induced upon HCMV infection, but this poses an intriguing question of why a virus would actively stimulate the expression of a protein that is known to negatively impact its replication. Interestingly, the evidence seems to show that HCMV possesses several elaborate strategies to not only subvert the viperin-mediated antiviral activity, but at the same time recruit this cellular enzyme to its own advantage by exaggerating its natural function to facilitate viral replication.

The first strategy of HCMV evasion is the encoding of a viral mitochondrion-localized inhibitor of apoptosis protein (vMIA), which is able to sequester viperin and translocate it to the mitochondria from the endoplasmic reticulum. In the mitochondria, viperin promotes β-oxidation of fatty acid, reducing ATP synthesis and disrupting the actin cytoskeleton, overall stimulating the production of viral progeny [64,65]. This may also potentially reflect a viral substrategy to create an inhibitory environment for viruses other than HCMV. Second, viperin enhances lipid synthesis in infected cells via transcriptional enhancement of mediators involved in metabolism of fatty acids, such as glucose transporter type 4 (GLUT4) and AMP-activated protein kinase (AMPK). As a result, it is followed by increased import of glucose and translocation of the glucose-activated transcription factor carbohydrate-responsive element-binding protein (ChREBP) to the nucleus, and later on, by increased lipid synthesis. This cascade of events finally leads to increased synthesis of the viral envelope and effective generation of infectious viral particles [66].

Overall, the evidence indicates that viperin acts as a potent metabolic regulator of HCMV-orchestrated modulation of lipid synthesis in the host, and should be considered as a potential target for therapeutic developments against HCMV.

Apolipoprotein B editing catalytic subunit-like 3 (APOBEC3)

The APOBEC3 (A3) family of proteins consists of seven members, namely A, B, C, D, F, G and H, which deaminate cytosine to uracil in single-stranded DNA and RNA substrates [67]. A3 proteins are widely recognized as essential players in the defense against viruses, particularly against HIV-1 [68] and other retroviruses, effectively introducing hypermutations into retroviral DNA during reverse transcription. However, recent findings suggest that A3 enzymes are also able to restrict the replication of several DNA viruses, such as HBV [69,70] and parvoviruses [71,72]. Besides, different A3 isoforms are able to edit the genomes of HPV [73] and BK polyomavirus (BKV) [74]. Genomes of

some herpesviruses, such as HSV-1 and Epstein–Barr virus (EBV), are deaminated by A3 on both strands [75]. The identification of new potential A3 targets is currently ongoing.

Recently, Weisblum *et al.* [76] reported the role of APOBEC3A (A3A) editing activity upon HCMV infection and its vertical transmission, and reported that A3A appears to act as a potent RF of HCMV replication both *ex vivo* in the human decidual tissues and *in vivo* in amniotic fluid samples obtained during natural congenital infection. Moreover, it is noteworthy that HCMV-mediated induction of A3A has not been detected in human fibroblasts, epithelial cells or chorionic villi in organ culture, which may suggest that upregulated A3A expression is most likely cell- and tissue-specific. The results of the study greatly contribute to greatly improve our understanding of the innate mechanisms acting to limit transplacental HCMV transmission. However, even though the results may shed light on important insights regarding the A3A's ability to restrict HCMV, many aspects regarding A3 specificity in different cells and tissues remain unresolved. For instance, it is not well established whether any other A3 proteins are induced by HCMV in other susceptible cells.

To address this matter, we have recently reported that APOBEC3G (A3G) is strongly upregulated in human foreskin fibroblasts (HFFs) infected with HCMV and its induction is IFN-β-mediated. However, both overexpression and gene knockout A3G did not demonstrate a restriction effect on viral replication in HFFs. Furthermore, we suggest that throughout evolution, under intense selective pressure, HCMV has shaped its genome nucleotide composition to avoid A3G-mediated restriction. This elaborate escaping strategy has been performed by limiting the A3G target motifs (CCC:GGG), particularly in genes essential for viral replication; whereas, no such pattern has been identified for the other target motifs of A3 family members [77]. Therefore, it could be interesting to further determine the role of other A3 members in distinct viral infections.

It is worth mentioning that not all DNA viruses seem to be susceptible to A3-mediated antiviral activity, for instance, vaccinia virus is not inhibited by APOBEC enzymes, potentially due to the incorporation of its replication complex in cytoplasmic bodies [78]. Considering this fact, it is possible that DNA viruses may escape APOBEC3 activity by encoding an undiscovered inhibitor, avoiding induction of A3 proteins, preventing entrapment into virions and/or replicating in privileged subcellular locations or, alternatively, in cells with reduced A3 levels [79].

Survival time-associated PHD protein in ovarian cancer 1 (SPOC1)

The cellular protein SPOC1, or PHD finger 13 (PHF13), was initially discovered as a cellular protein with a PHD domain, elevated expression levels of which in epithelial tissues correlated with unresectable carcinomas and decreased survival rates of ovarian cancer patients. Later studies reported that SPOC1 is a multifunctional protein, associated with the modulation of several vital processes, including development [80], cell proliferation [81] and DNA damage response [82,83], acting as a potent regulator of chromatin structure [81,83]. It has been proposed that the SPOC1–chromatin interaction occurs through a C-terminus-located PHD, which in its turn, senses histone marker H3K4me2/3, enabling SPOC1 binding. Upon binding, SPOC1 triggers compaction of the chromatin by recruiting histone methyltransferases (HMTs), in other words, SETDB1, GLP or G9A, which eventually leads to an increase of repressive H3K9me3 [83]. Although the PHD domain demonstrates a specific binding affinity to H3K4me2/3, it seems to be relatively weak, indicating that additional stabilizing chromatin interaction may occur to ensure the binding. In line with this hypothesis, there is additional evidence of SPOC1 directly binding DNA via a domain located centrally, simultaneously with chromatin-affiliated polycomb repressive complex 2 (PRC2) and RNA Pol II; thereby, acting in a multivalent fashion [84]. This feature of direct binding to DNA as well as H3K4me2/3, together with the indirect binding of other chromatin-affiliated proteins, stabilizes weak H3K4me2/3 interactions and enhances SPOC1-chromatin complex avidity. Presumably, this ability plays an additional beneficial role in DNA damage response [83], as it has been recruited to DNA double-strand breaks in an ataxia-telangiectasia mutated (ATM)-dependent manner.

In addition to its cellular regulatory functions, SPOC1 additionally contributes to the intrinsic defense against viruses [85]. As described by Schreiner *et al.*, levels of SPOC1 decreased in cells infected with human adenovirus type 5 (HAdV5), attributed to proteasomal degradation early after infection, which is mediated by the HAdV5 E3 ubiquitin (Ub) ligase complex E1B-55K/E4orf6 [85]. Moreover, the same study provided evidence that overexpression of SPOC1 resulted in decreased viral DNA and protein synthesis, reporting that restriction of virus infection occurred at the transcriptional level, while SPOC1 depletion led to increased virus titers [85].

In a recent study, Reichel *et al.* [86] addressed the way by which SPOC1 contributes to HCMV infection. Interestingly, in contrast to HAdV5 and HIV-1 infection, they have demonstrated that SPOC1 protein level is enhanced upon early steps of HCMV infection, whereas in late replication phase it degrades in a glycogen synthase

kinase 3β-dependent manner. Furthermore, the overexpression of SPOC1 in fibroblasts negatively impacted viral replication, while depletion of SPOC1 resulted in increased level of IE gene products. It is worth noting that SPOC1 associates with the HCMV MIEP region, supporting the scenario of SPOC1-induced silencing of viral IE expression via epigenetic modifications [86].

Myxovirus resistance (Mx)

The Mx proteins are interferon-inducible dynamin-like large GTPases that play a significant role in innate immune defense by exhibiting a potent activity against numerous RNA and DNA viruses [87,88]. Two human genes, *MX1* and *MX2* encode the MxA and MxB proteins under the direct control of IFN I and III. Initially, MxA demonstrated a broad spectrum of antiviral activity against RNA viruses, such as influenza A viruses (IAV), vesicular stomatitis virus (VSV) and measles virus [87], while the function of MxB had remained unknown until recently, when it was defined as a potent inhibitor of HIV-1 [89–92]. In this regard, Mitchell *et al.* [93] analyzed *MX2* evolution in primates, suggesting that MxB has a broader spectrum of antiviral activity extending beyond retroviruses.

Recently, MxB has been described blocking the replication of murine γ-herpesvirus 68 (MHV68), a member of the γ-herpesvirus family. Schilling *et al.* [94] have expanded the study of the antiviral activity of MxB to a wider range of herpesviruses, reporting that MxB acts as an efficient pan-herpesvirus RF in a manner distinct from its relative protein family MxA. In this study, MxB protein has demonstrated its high efficiency in restriction of herpesviruses of all three subfamilies, including HCMV, by targeting early viral gene expression. However, the molecular mechanisms of MxB inhibitory activity remain unclear. It is currently assumed that MxB takes on an antiviral conformation that enables recognition and restriction of the herpesviruses, through targeting of the viral capsids that accumulate at the nuclear pore and/or affecting components of the nuclear pore complexes (NPC), thus hindering viral uncoating. Recent study supported this idea by showing a defect in the viral DNA delivery into the nucleus of HSV-1-infected cells, suggesting the role of MxB as a 'cytoplasmic gatekeeper' against herpesviruses [95].

It remains to be further established whether HCMV encodes a viral MxB antagonist or employs any other strategy to counteract MxB.

Cytomegalovirus immune evasion strategies

HCMV pathogenesis is driven by a complex bidirectional relationship between HCMV and the immune system. In this way, HCMV targets the essential components of the innate immune system: pro-inflammatory NF-κB and interferon signaling pathways through numerous antagonizing and modulatory genes. Here we discuss evasion strategies employed by HCMV to alter interferon (Figure 2) and NF-κB (Figure 3) signaling pathways to assure successful viral replication and persistence.

HCMV evasion of the interferon response

Once the pathogen has been detected, intracellular pattern recognition receptors (PRRs) stimulate cascades of events resulting in the activation of numerous transcription factors, such as NF-κB, mitogen-activated protein kinases (MAPKs), IRF3 and IRF7, which mediate the transcriptional induction of interferons, and subsequently, the production of pro-inflammatory chemokines that lure immune cells to the site of infection [96,97]. Interferons are a subset of cytokine molecules classified into three distinct groups, namely type I IFN, type II IFN and type III IFN, that regulate a wide range of vital processes, including cell proliferation, autoimmunity, apoptosis, cancer and defense against viral infections [96,98].

Undoubtedly, upon HCMV infection, the interferon response appears as a complex phenomenon, activated by various mechanisms and at different time points during viral infection. The interferon response is activated immediately following initial viral sensing. A significant and growing number of newly identified cellular sensors, activated upon HCMV binding and entry, aim to detect the invader. So far, the main proteins involved in viral genomes detection are the toll-like receptor-2 (TLR2) and CD14, interacting with the envelope proteins gB and gH, along with dsDNA sensors, such as Z-DNA binding protein 1 (ZBP1) [99], TLR9 [100] and cGAS [100]. In this cellular environment, the virus developed multiple interferon evasion strategies (Figure 2).

In addition to IFI16 and viperin as discussed above, HCMV also exploits a family of interferon-stimulated genes (ISGs), named interferon-induced transmembrane proteins (IFITMs) to facilitate its replication. This happens in the very late stages of infection, such as virion maturation and assembly, where IFITMs are required for the optimal establishment of virus assembly complex [101]. Moreover, unlike what was observed for RNA viruses [102],

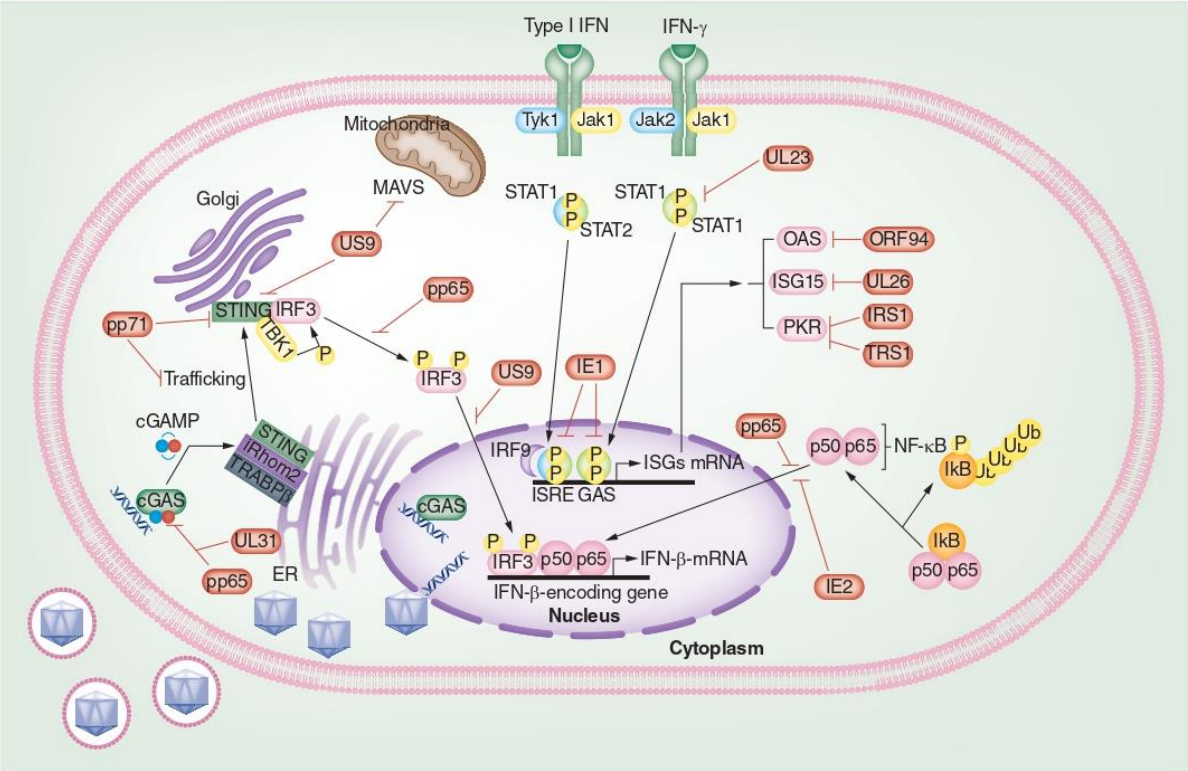

Figure 2. Schematic representation of the human cytomegalovirus evasion strategies from interferon antiviral activity.

the HCMV entry step is not affected by overexpression of IFITM1, 2 and 3 [103], supporting a proviral role for these proteins.

Results from several groups [62,104–106] have demonstrated that viral pp65 is able to inhibit the type I IFN response; however, it remains unclear at what level pp65 blocks the activation of interferon. Browne *et al.* [105] have shown that pp65 inhibits interferon-responsive genes by blocking IRF1 and NF-κB activation. In contrast, other work [104] has shown that pp65 drives dephosphorylation of IRF3 along with its nuclear export [107]. Last, recent work by Biolatti *et al.* [62] provided evidence that pp65 is binding cGAS and inhibiting the cGAMP release; thereby, preventing its cooperation with STING and impairing the cGAS/STING pathway. In addition, Huang *et al.* [108] have demonstrated that HCMV protein UL31, similarly to pp65, acts as an inhibitor of cGAS. Specifically, they showed that UL31 actively promotes dissociation of DNA from cGAS, resulting in inhibition of cGAS enzymatic activity and reduced cGAMP production.

In addition, HCMV tegument protein pp71 (pUL82) also contributes to immune evasion by disrupting the STING–iRhom2–TRAPb pathway [109].

Choi *et al.* [110] have described the ability of viral US9 protein to block IFN-β by targeting both STING–TBK1 signaling and mitochondrial antiviral-signaling protein (MAVS) during late HCMV infection. In greater detail, US9 disrupts oligomerization of STING and STING/TBK1 assembly via competitive interaction, thus inhibiting the nuclear translocation of IRF3 and secretion of IFN-β. The study has demonstrated that deletion of the US9 C-terminal domain diminishes its ability to weaken the STING- and mitochondrial antiviral-signaling protein-mediated interferon responses, emphasizing the critical role of US9 C-terminal region function in immune evasion [110].

In addition, several studies have reported that the HCMV IE2 protein affects the IFN-β production by preventing the NF-κB binding to the IFN-β promoter [111–113]. Consistent with these data, cells expressing IE2 have shown decreased levels of STING [114]. This decrease suggests that IE2 affects STING in order to block IFN-I signaling [114].

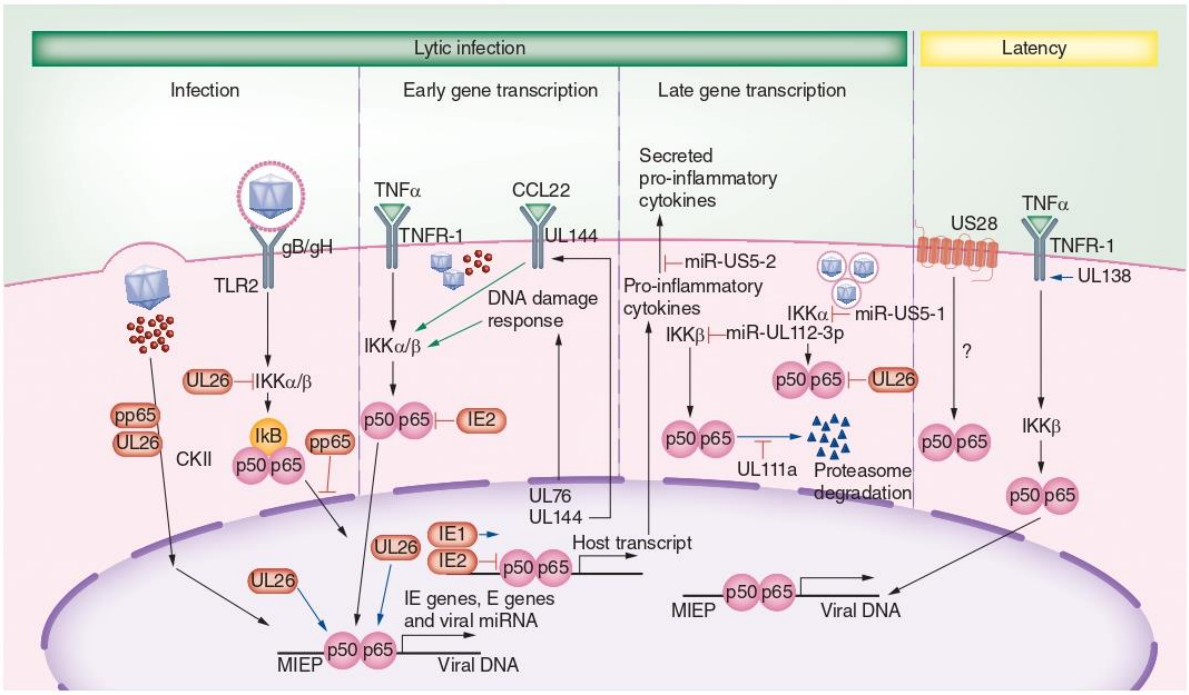

Figure 3. Model depicting the modulation of the NF-κB signaling pathway by human cytomegalovirus.

Finally, HCMV tegument proteins also impact the modulation of the type II IFN, which is generally less well-characterized than HCMV-mediated impact on type I IFN. In greater detail, it is well established that upon viral infection IFN-γ activates Janus kinase/signal transducers and activators of transcription (JAK–STAT) cellular pathways. STAT1, an essential transcription factor that binds and activates transcription at promoters containing γ-activated sequence (GAS) elements, regulates the transcription of type II IFN-dependent genes. After IFN-γ binding to its receptor, JAK1 and JAK2 become activated and regulate the downstream phosphorylation of STAT1. This results in STAT1–STAT1 homodimers, which are later transported to the nucleus where they bind GAS elements, resulting in an induced transcription of ISGs [115]. In this context, human N-myc interactor (Nmi) protein is an interactor of STAT1, essential for the activation of STAT1-dependent transcription induced by IFN-γ. Interestingly, recently Feng *et al.* [116] have demonstrated that viral protein UL23 specifically interacts with Nmi, inhibiting Nmi translocation into nucleus along with its associated protein STAT1, resulting in a reduced IFN-γ expression and promotion of viral resistance to IFN-γ. In line with this hypothesis, the blocking of UL23 expression resulted in higher transcription of IFN-γ stimulated genes and significant decrease of virus production [116].

In parallel, there is significant effort to understand the relationships between individual interferon genes and tegument proteins. For instance, cellular ISG15 encodes a Ub-like protein that is able to bind cellular and viral proteins in a Ub-similar manner. Several studies proposed ISGylation as an antiviral mechanism during early stages of infection acting through cGAS-STING viral DNA sensing, resulting in inhibition of HCMV replication [117,118]. As predicted, HCMV US26 protein has recently been shown to interact with cellular ISG15, along with several proteins that enable ISG15 activation and its binding to the target proteins [117]. However, many questions about these interactions and their contribution to the infection outcome remain open.

Host cell cytosolic proteins are able to sense not only DNA, but also dsRNA and mount similar responses. For example, protein kinase R (PKR) signaling [119] can trigger several immune responses, including type I IFN production [120] and NF-κB activity [121]. Also in this context, HCMV is able to counteract these antiviral measures by means of two IE gene products, IRS1 and TRS1. A study by Marshall *et al.* [122] has shown that deletion mutants of IRS1 and TRS1 (individually and in tandem) do not alter viral growth. In contrast, IRS1/TRS1 double deletion mutants are characterized by a significant reduction of protein synthesis and replication in HFF [122].

Yet, Ziehr *et al.* [123] reported that infection outcome correlated with levels of PKR activation, as demonstrated by the observation that in cells silenced for PKR, viral growth is restored upon concurrent loss of IRS1 and TRS1, confirming the role of IRS1 and TRS1 in interferon modulation.

To summarize, HCMV has evolved sophisticated mechanisms to modulate the host interferon response. The latest evidence contributes to our understanding of the molecular mechanisms employed by HCMV to successfully evade host innate immune responses. Better understanding of these mechanisms may greatly assist in future development of therapeutic interventions to treat autoimmune diseases characterized by the chronic overproduction of cytokines, including type I IFN.

HCMV & NF-κB signaling

The NF-κB signaling modulates different aspects of innate and adaptive immune response and can be activated by a plethora of stimuli, including HCMV. As discussed below, a number of HCMV proteins are associated with NF-κB modulation.

Upon HCMV infection, the modulation of essential cellular signaling pathways begins once viral tegument proteins are being released and disseminated in host cytoplasm. The viral pp65 protein, as mentioned previously, plays a role in interferon inhibition during early steps of HCMV infection that has not yet been completely elucidated. Besides the interferon modulatory role, it has been suggested that pp65 may act as a potent regulator of the NF-κB pathway. Indeed, work by Browne *et al.* [105] has demonstrated that use of a pp65-deletion HCMV mutant leads to an increase of NF-κB target genes, stimulating the nuclear binding activity of NF-κB transcription factors. However, it remains unknown how exactly pp65 modulates NF-κB or whether its modulation of interferon and NF-κB networks may be functionally related.

Much evidence supports the hypothesis that another HCMV tegument protein, UL26, can impact NF-κB activity, even if the exact mechanism of this inhibition remains to be established: a UL26 deletion mutant virus displays an attenuated phenotype; UL26 blocks the I kappa B kinase (IKK) complex phosphorylation and NF-κB translocation [124,125] and UL26 expression is sufficient to block TNFα-mediated NF-κB activation [124,125]. Although UL26 is a tegument protein, it seems that it is unable to block the activation of NF-κB upon early stages of infection, but rather has a strong effect during the late infection, when it is detected in cytoplasm, in contrast to the early stage when it is nuclear [126]. At the same time, it is worth mentioning that it cannot be ruled out that an interaction between UL26 and NF-κB may occur early during infection, because viruses deficient for UL26 display higher sensitivity to a challenge with TNFα [124].

Conversely, other HCMV tegument proteins are able to induce NF-κB signaling, which favors viral replication. Among these, UL76, a viral tegument-associated endonuclease, is able to activate the canonical NF-κB pathway via DNA damage response; thereby, inducing IL-8 release, which depends on the cellular ATM and IKKβ kinases [127]. In this regard, induction of IL-8 displays a critical role upon HCMV infection as neutrophils, primarily attracted by IL-8, are involved in virus dissemination. However, the same study indicates that upon HCMV infection, besides *UL76*, other genes may be responsible for the stimulation of IL-8 expression, partly through activation of ATM. HCMV UL76-deletion mutants have demonstrated a strong growth defect [128], but it remains unknown whether this attenuation is related to an increased IL-8 production.

It is known that several cellular mRNAs and proteins become incorporated into HCMV virions [129,130]. Potentially, some of these cellular proteins could also be modulating NF-κB signaling together with viral factors. For instance, casein kinase II (CKII) has been detected in the viral tegument and has been shown to activate NF-κB through phosphorylation of the IκB. This phosphorylation leads to the release of associated NF-κB subunits in the nucleus and the induction of NF-κB-dependent transcription [131].

The HCMV IE proteins also contribute to the control of the NF-κB signaling. For instance, IE1 acts as a potent transactivator of NF-κB constituents and their downstream targets; moreover, it upregulates p65, TNF-α, IL-6 and IL-8 and increases NF-κB binding activity [132]. Furthermore, UL144, an IE TNF-receptor-like transmembrane receptor [133], activates the expression of the immune cytokine CCL22 by interacting with TNF receptor associated factor 6 (TRAF6) in perinuclear regions, thus enabling NF-κB transcription factor translocation and binding [134]. In support of these data, it has been demonstrated that siRNA targeting UL144, TNF receptor associated factor 6 or NF-κB negatively impacted downstream CCL22 expression stimulated by HCMV [134]. The CCL22 cytokine is a key chemoattractant, able to recruit Th2 and regulatory T-cells; thereby, mediating adaptive immune responses [134]. Moreover, IE2 inhibits NF-κB signaling during all phases of HCMV infection either by preventing NF-κB subunit

dimer interactions or blocking it with specific NF-κB target promoters, such as IL-6 [113,135]. Notably, at the same time, the antagonistic effects of IE2 do not block NF-κB induction by UL144 [136].

To summarize, there is strong evidence that the virus uses an elaborate strategy to provide itself a thriving pro-inflammatory environment, where NF-κB transactivates the MIEP at the early stages of infection, while at the same time keeping a low profile to stay undetected by the host immune system and thus avoiding triggering a broader immune response [137].

While NF-κB signaling is activated early in HCMV infection, in the late stages the HCMV effect changes to an inhibitory mode, increasing expression of the genes that antagonize NF-κB activity. For instance, UL111a, or cmvIL-10, named after its functional similarity to the human cytokine IL-10, exerts its inhibitory activity on both NF-κB and interferon signaling, inhibiting IL-10 receptor binding and IκBα degradation [138–140].

The exact mechanisms and signals that lead to the switch of an HCMV infection from the limited lytic phase to the prolonged latency phase, as well as back to spontaneous reactivations remain only partially understood, although the processes of immunosuppression and inflammation are believed to contribute [141]. In line with this hypothesis, several studies indicate that HCMV genes activate the NF-κB network upon reactivation [142] via NF-κB stimulation of MIEP expression [143]. One of the viral proteins involved in MIEP modulation is the viral chemokine receptor US28. During the lytic phase of HCMV infection, it is expressed early [144], but it also represents one of the complex array of viral proteins expressed during latency [145]. It has been suggested that US28 activates MIEP via the NF-κB pathway: upon latency, US28 expression activates the MIEP; thereby, assisting reactivation. In greater detail, US28 promotes constitutive NF-κB activation via interaction with the Gq/11 family of G protein, mediating the Gβγ subunits release that stimulates downstream NF-κB activity [146]. US28 is also an important player of HCMV latency, likely by NF-κB modulation. Indeed, mutants lacking US28 are able to restore the lytic cycle and HCMV-infected cells are efficiently targeted by T-cells [147].

Another HCMV protein involved in interferon pathway modulation is UL138, expressed during latency, that activates and stabilizes the cell surface expression of TNFR1 [148]. A recent study by Lee *et al.* [149] has shed light on UL138's role in maintaining HCMV latency: in addition to UL138 promotion of the sensitivity to TNFα in latently infected cells, UL138 strongly represses MIEP transactivation by blocking the interaction between MIEP and cellular demethylases [149].

Along with modulatory proteins, HCMV also employs numerous miRNAs that interfere with the NF-κB network. The virus encodes 26 miRNAs that are involved in modulation of several vital cellular processes, including cytokine production, vesicle transport and immune signaling. Viral miRNAs begin to accumulate during the early stages of infection, reaching peak expression at the later time points [150–152]. MiR-US5-1 and miR-UL112-3p, encoded by HCMV, have been shown to avert NF-κB cytokine signaling by downregulation of IKKα and IKKβ kinases [132]. In addition, miR-US5-2 has been found to block secretion of cytokines in infected cells, thus terminating the positive feedback loop of NF-κB activation [152]. MiR-UL148D, a miRNA that is highly expressed during latent infection, has been shown to inhibit NF-κB upstream adapters and repress the production of IL-6; thereby, permitting the infected cell to escape immune surveillance [153].

To summarize, HCMV utilizes several distinct strategies to regulate the NF-κB pathway and appears as an interesting paradox, reflected in multiple molecular interactions, complex virus–host interplay and regulation of multiple aspects of NF-κB signaling during different steps of infection. In this way, both HCMV proteins and viral miRNAs have been shown to block NF-κB signaling, activating constituents of the NF-κB pathway to facilitate lytic replication or induce reactivation from latency. That clearly suggests that NF-κB signaling is involved in multiple transcriptional scenarios depending on specific upstream stimuli and specific viral manipulations. To date, no unifying theory explains all the reported functional aspects and; therefore, our understanding of HCMV-mediated modulation of NF-κB is incomplete. Further efforts are required to better understand the dynamics and mechanisms of such immunomodulation, especially in different biological scenarios of HCMV infection, including viral dissemination, persistence, pathogenesis, latency and reactivation.

Future perspective

Significant progress has been made in the last few years in our understanding of the pathogenesis and diagnosis of HCMV infection. However, HCMV remains an unsolved matter of high clinical importance for many, as the currently available drugs fail to successfully eliminate the infection. Considering the profound effects of HCMV infection on the health and quality of life of immunosuppressed individuals, the elderly and congenitally infected children, the development of a vaccine against congenital HCMV and therapeutic approaches to control HCMV

disease remain a high priority. The lack of understanding of the complex interplay between HCMV and innate immunity, involving multiple pathways and viral modulation strategies, is partially to blame for the current absence of effective vaccines or therapeutics.

In this review, we reviewed the overall story of how innate immune players cooperate with each other to counteract HCMV infection, with particular focus on host RFs, interferon and NF-κB signaling. In addition, we attempted to address the various evasion strategies and mechanisms that the virus relies on to escape immune surveillance. Over the last few years, a wide panel of cellular proteins implicated in resisting HCMV have been uncovered and assessed. The number of new studies reporting how cellular factors already known to be involved in a variety of vital cellular processes also display antiviral activity against HCMV, rises yearly. Therefore, one may speculate that illuminating works toward the identification of novel RFs of HCMV infection, as well as broader insights into the function of the previously reported ones, will be undertaken in the nearest future.

Simultaneously, we expect new reports to shed light on the remarkable ability of HCMV to evade the intrinsic immune system and detailing the exact strategies that the virus employs to do so. Given the large numbers of functional HCMV proteins, identification and characterization of those that target a certain host RF may represent a challenging, but at the same time, rewarding avenue of investigation.

Thorough understanding of the molecular interactions between HCMV and RFs may provide a solid platform for the future development of therapeutic interventions designed to target the viral immune modulators directly. We may speculate that those therapeutics targeting the early steps of the HCMV infection could avert viral attempts to exploit the host metabolism or immune mediators to its own advantage, thus enhancing the immunocompetence of the host.

Finally, the intriguing interplay between HCMV and host immune signaling cascades represents a wide platform for future discoveries. The dynamics and tuning of different cascade components by HCMV in a variety of ways and in different contexts of infection represents a field of unresolved work. It is of great clinical importance to further elucidate the roles of immune restriction and HCMV countermechanisms in determining the final outcome

Executive summary

Human cytomegalovirus

- Human cytomegalovirus (HCMV) is a widely spread opportunistic pathogen that causes serious disorders in newborns and immunocompromised adult patients.
- There are currently no vaccines against HCMV infection and only few antiviral drugs are recommended for treatment, which are limited by their low efficacy, high hematopoietic toxicity and poor bioavailability.

Immune modulation

- HCMV represents a paradigm for viral immune evasion. It encodes numerous proteins with potent immunomodulatory functions and profoundly affects the host immune response.

HCMV restriction factors

- Restriction factors represent a frontline defense against HCMV infections. The γ-interferon-inducible protein 16 (IFI16), viperin, apolipoprotein B editing catalytic subunit-like 3 (APOBEC3) and survival time-associated PHD protein in ovarian cancer 1 (SPOC1) are the restriction factor (RFs) that strive to hold HCMV infection back.

Evasion from the interferon response

- HCMV has evolved many strategies to escape the innate immune response: the HCMV immediate-early (IE) proteins IE1 and IE2 counteract antiviral cytokine production, while HCMV tegument proteins impact the activation of the type I–II IFN response.
- HCMV pp65 acts as the key inhibitor of the IFN-I, preventing the activation of NF-κB and IRF3 and impairing the cyclic GMP-AMP synthase (cGAS)/STING signaling pathway.

Modulation of NF-κB signaling

- HCMV triggers the expression of both agonists and antagonists of NF-κB signaling in order to assist viral replication, dissemination, latency and reactivation.
- Antagonists: a number of HCMV proteins and miRNAs are able to inactivate the IKK complex or downstream binding of the NF-κB transcription factor to its target sequences to avoid induction of antiviral and pro-inflammatory genes activated after virus binding and entry.
- Agonists: induction of the NF-κB signaling pathway upon early stages of HCMV infection activates expression from the MIEP, thus initiating the lytic cascade of gene expression.

Future perspective

- Development of new antiviral strategies targeting the innate immune response to achieve protection for immunosuppressed transplant patients and to prevent congenital infections.

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of HCMV infection, as doing so opens new horizons in the development of effective therapeutic agents, targeting HCMV during both the lytic and latent phases.

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