UNIVERSITY OF TURIN

PhD school in Life and Health Sciences

Molecular Medicine

Characterization of citrullination during infection with DNA and RNA viruses: a new strategy for host-targeting antivirals drugs

Selina Pasquero

UNIVERSITY OF TURIN

PhD school in Life and Health Sciences

Molecular Medicine

XXXIII Cycle

Academic Years 2020-2021

Characterization of citrullination during infection with DNA and RNA viruses: a new strategy for host-targeting antivirals drugs

Tutor: Prof. Santo Landolfo (2017-2020)
Dr. Valentina Dell'Oste (2020-2021)

Candidato: Selina Pasquero

Coordinator: Prof. Francesco Novelli

CONTENTS

ACKNOWLEDGEMENT……………………………………………………………………………4

1. Citrullination

1.1 The post-translational modification …………………………………………………....5

1.2 The PAD family………………………………………………………………………..5

1.3 PADs' genetics…………………………………………………………………………6

1.4 The tissue specificity and biological function of PADs………………………………..6

1.5 Citrullination and disease………………………………………………………………7

1.6 PADs inhibitors………………………………………………………………………….10

2. Herpesviruses

2.1 The Herpesvirus Family…………………………………………………………………12

2.1.1 Virion structure…………………………………………………………...13

2.1.2 Genome organization……………………………………………………….13

2.1.3 Viral replication and antiviral target…………………………………………...14

2.2 HCMV

2.2.1 The Human Cytomegalovirus…………………………………………………15

2.2.2 HCMV and autoimmune disease………………………………………………17

2.3 HSV

2.3.1 The Herpes Simplex Virus……………………………………………………18

2.3.2 HSV1 and Alzheimer…………………………………………………………19

3. Coronavirus

3.1 The coronaviridae family……………………………………………………………..20

3.2 Antiviral drugs………………………………………………………………………..21

REFERENCES………………………………………………………………………………24

PUBLICATIONS……………………………………………………………………………36

ACKNOWLEDGEMENT

I would like to sincerely thank my supervisors Prof. Santo Landolfo and Valentina Dell'Oste. I also thank the co-supervisor Prof. Marco De Andrea. I thank them for giving me the opportunity to join the lab, for their time, advice and fruitful discussions.

Also my deepest gratitude goes to ALL members of Viral Pathogenesis Lab (VIPLab) who patiently shared advice, supplies, techniques, joys and sorrows of everyday lab life with me. I am especially thankful to Dr. Gloria Griffante, Dr. Francesca Gugliesi and Dr. Matteo Biolatti for the inspiration they were -and still are- to me, for their enthusiastic and professional work, encouragement, collaboration and ability to solve whatever obstacles arose.

I also thank my very supportive family. I would like to thank them for providing me with much needed support during hectic times, for supporting my decisions, for their patience and unconditional love.

1. Citrullination

1.1 The post-translational modification

Citrullination, also called deamination, is a post-translational modification (PTM) in which the guanidinium group of a peptidyl-arginine is hydrolyzed to form peptidyl-citrulline, a non-genetically coded amino acid. The conversion of arginine to citrulline is catalyzed by a family of calcium-dependent enzymes called Peptidyl arginine deiminase (PADs) and results in an increase in molecular mass of 0.984 Da and the loss of a positive charge for each converted arginine[1]. This change determines a substantial variation in the acidity characteristics of the site within the amino acid chain, thus changing the isoelectric point (pI) from 11.41 for arginine to 5.91 for citrulline. This conversion can affect the molecule's ability to create hydrogen bridges and thus modify its interactions both intra and intermolecular. The protein can therefore undergo conformational changes, which can lead to functional alterations and variations in the half-life of the protein[2].

1.2 The PAD family

Peptidyl arginine deiminase (PADs) are a family of cellular enzymes that catalyze the citrulline reaction, that is the calcium-dependent deamination of an arginine residue in a citrulline one.

Since there are no codons or tRNAs encoding the amino acid citrulline, this cannot be incorporated during the synthesis of a new protein chain; therefore, when in 1958 citrullinated proteins were identified in mammalian tissues, the existence of enzymes capable of converting arginine residues into citrulline was predicted. As expected, in 1977 these enzymes were identified, which were subsequently purified from the tissues of numerous vertebrate species, from fish to human[3].

In addition to the eukaryotes, only one prokaryote was found capable of expressing a member of the PAD family, *Porphyromonas Gingivalis*, the main pathogen responsible for periodontitis. This enzyme differs from eukaryotic PADs in that it is not dependent on the calcium ion but is activated by high pH levels. Despite these differences, the action of this enzyme could be the basis of a correlation between bacterial infection and the onset of RA[4,5].

In mammals the PAD family consist of five members, PAD 1-4 and PAD 6, all of the above isoforms are unable to convert free L-arginine into L-citrulline and require calcium ion as a cofactor. Intracellular calcium levels in normal physiological conditions are not sufficient to activate PADs, thus suggesting that citrullination is related to phenomena in which an alteration of calcium homeostasis is present[6].

1.3 PADs' genetics

Numerous studies have shown high conservation in the sequence of PADs. In the same species, the different isoforms have 50-55% of the amino acids in common, while the sequence homology of each individual isoform between the different species is even higher, from 70 to 90%[3].

This means that the gene length, the number of exons, and the molecular weight are very similar between isoforms (Table 1). Moreover, the isoelectric point of all the isoforms is almost the same, the average pI value for human PAD is 5.8, which leads the enzyme to have a net negative charge in physiological conditions. This feature is important in determining the interaction with the positively charged substrate, the arginine residues, and with the calcium ion, the cofactor capable of binding the enzyme in triplicate in three low-affinity sites[7].

All five isotypes of the enzyme are located in a single gene cluster, located in humans on the short arm of chromosome 1 where it extends for 334 Kb. Moreover, within mammalian genome, the PAD isoform encoding genes are ordered the same, from PAD2, 1, 3, 4 to 6. This consideration, coupled with the high sequence homology, suggests that mammalian PADs are the result of a series of gene duplication events in a common ancestor[3].

1.4 The tissue specificity and biological function of PADs

The biggest differences between the five PAD isoforms are tissue expression specificity and substrate specificity, on the basis of these unique features PADs exert their unique biological function.

PAD 1 is mainly expressed in the epidermis and uterine cells. PAD1-mediated citrulline regulates the content of keratin in the body. Keratin plays a connective and protective function during physiological processes, and controls mammals' hair and toenail health[8]. In addition, PAD1 regulates transcription through citrullination of the histones H4 R3 and H3 R2/8/17 in early stages of embryonic development and promotes the production of 2- or 4-cell embryos[9,10].

PAD2 has been found in muscle tissue, the central nervous system, and hematopoietic cells including mast cells and macrophages. In the CNS, including neurons, glial cells, astrocytes, microglia, and oligodendrocytes PAD2 is highly expressed[3,11,12]. For that reason, this isozyme plays an irreplaceable role in the CNS. It can mediate the formation of citrulline in myelin basic protein (MBP)[13]. MBP can bind to proteins in the neuronal plasma membrane, while citrullinated MBP disrupts the arrangement of lipid-proteins in the membrane. This structural disruption increases protease hydrolysis, which eventually leads to the disintegration of myelin sheath in the CNS.

PAD3 is expressed in the developing nervous system and neural stem cells and is also highly expressed in the epidermis and hair follicles[14]. PAD3 and PAD1 have similar functions in controlling

the health of hair, toenails, and other tissues by regulating the amount of keratin. PAD3 can target and regulate the interaction between keratin intermediate filaments and filamentous aggregates on the lower epidermis and maintain the steady state of the human epidermal cuticle[15].

PAD 4, also known as PAD5, has been found in neutrophils and eosinophils, in the spleen and secretory glands and is the only isoform localized in the cell nucleus[16]. PAD4 mutually influences the expression of estrogens and p53 target genes and could therefore be involved in tumorigenesis processes. Furthermore, the citrullination operated by PAD4 participates in the formation of the extracellular antibacterial traps of neutrophils (NETs), this is involved in the generation of new autoantibodies[17,18].

Finally, PAD6 is expressed in ovaries and early embryos and plays and indispensable role in embryonic development[19,20].

1.5 Citrullination and disease

The function of the five PAD isoforms has been studied in different physiological and pathological conditions. PAD1, physiologically involved in the keratinization of the epithelia, is hypofunctional in psoriasis[10]. PAD2, essential for myelin sheath stability and brain plasticity, as such, this enzyme is involved in the occurrence and development of neurodegenerative diseases[21]. PAD4 is the PAD isoenzyme that mainly translocates into the nucleus and citrullinates histones. In that way this enzyme regulates the gene expression and the formation of NETs, so its dysregulation is involved in many autoimmune diseases and tumors[18,22,23].

Overall, aberrated citrullination has been mainly related to autoimmune diseases, cancers and neurodegenerative diseases.

Citrullination and autoimmune disease. The changes in the protein structure induced by citrullination can generate new epitopes with immunogenic capacity, that is, they are potentially able to activate an immune response against the antigen itself (autoantigen)[6,24]. In recent years, great attention has been given to citrullination due to its role in the induction of antibodies to citrullinated proteins (ACPA), a class of autoantibodies with diagnostic, prognostic and predictive value in rheumatoid arthritis (RA)[1,25,26]. Although rheumatoid arthritis is the pathological picture most correlated with the presence of antibodies to citrullinated proteins, an aberrant activation of the citrullination process can be observed during the course of various inflammatory diseases such as systemic lupus erythematosus, neoplasms and degenerative diseases[27]. Furthermore, citrullination appears to be involved in numerous cell death pathways such as apoptosis, autophagy and NETosis; these pathways are combined by an increase in intracellular calcium with respect to physiological conditions.

It has been shown that some environmental factors, such as cigarette smoke or air pollution nano materials, are able to induce citrullination processes in lung cells before the inflammatory response can be detected[28]. This suggests that aberrant citrullination could be regarded as an early sign of cell damage.

Citrullination and tumors. PAD enzymes play a vital role in regulating many vital cellular processes; therefore, it is necessary to maintain a perfect balance in the activity of these enzymes. Due to this delicate balance it is not difficult to imagine that neoplastic diseases associated with dysregulation of PAD and abnormal levels of citrullination are possible[29]. In particular, PAD4, by citrulling histones in the cell nucleus, acts as a regulator of gene expression. PAD4 was overexpressed at both the mRNA and protein levels in 12 types of tumors including malignant tumors of the breast, lung, liver, esophagus, colorectal, kidney, bladder, and ovarian cancers[18]. Interestingly, in benign tumors and non-cancerous inflamed tissue samples, PAD4 is not expressed, with the exception of gastrointestinal tract tissue samples. These higher levels of PAD in the tumor compared to non-tumor tissues suggest that the abnormal activity of PAD can induce a dysregulation of gene expression and when tumor suppressor genes are the targets of this dysregulation, tumor transformation can occur. Furthermore, PAD4, expressed in an unregulated way, interferes with the apoptotic process through the suppression of p53 and the citrullination of cytokeratin filaments essential for the creation of apoptotic bodies; this block of apoptosis favors the onset of neoplasms[17]. Recent studies have shown that inhibition of PAD4 enzyme activity can be used as a target for cancer treatment[30,31].

The PAD2 isoform is also involved in tumorigenesis processes through the citrullination of histone H3 and R26. These changes are associated with increased expression of HER2 and more than 200 genes under the control of the estrogen receptor. Furthermore, PAD2 levels are elevated in breast cancer cell lines suggesting its close involvement in breast cancer biology[32,33].

Citrullination and neurodegenerative disease. In recent years, it has been discovered that abnormal activation of the PADs causes a large number of citrulline-containing proteins to accumulate in patients with various neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease, multiple sclerosis (MS), and Huntington's disease, suggesting that excessive citrullination of proteins is involved in the onset and development of these diseases[34,35].

Antibodies against myelin basic protein (MBP) are detectable in serum of patients with active demyelinating lesions in MS[36]. Analogous to MS as an autoimmune condition, damaged myelin interacts with Aβ deposits in AD and antibodies to glial derived antigens are reported[37]. Acharya et al. suggested that autoantibodies in AD associate with host PAD4 and protein citrullination and

confirmed citrullination of pyramidal neuronal intracellular proteins in the AD hippocampus[38]. Matsuomi et al. found an abnormal accumulation of citrullinated proteins and an increase of the PAD2 content in the hippocampi of AD patients[39]. Moreover, it was found that the most over-citrullinated proteins in AD were structural proteins such as vimentin, MBP and the glial fibrillary acidic protein (GFAP)[21,40]. Noteworthy, Thompson et al. showed that human PAD3 (PADI3) is a key player in hNSC homeostasis, and particularly in caspase 3 independent cell death induced by increased intracellular calcium in hNSCs. This study demonstrates that PAD3 is an upstream regulator of Ca2+-induced cell death in human neural cells and sheds some light on the mechanisms involved. Furthermore, it highlights differences in the role of PAD2 and PAD3 in hNSCs[41]. Their findings in a human model identify PAD3 pathway targeting as a novel approach to reducing neural tissue loss in human pathologies where increased intracellular Ca2+ greatly contributes to cell damage.

Citrullination and viral infections. In recent years, numerous researches have been conducted on anti-CCP antibodies, through ELISA tests in patients with RA, and these have proved to be more specific and predictive than rheumatoid factor (RF), to the point of assuming the function of prognostic markers for the diagnosis of the disease in the early stages. Recent studies have revealed through ELISA tests the presence of anti-CCP antibodies also in some infectious diseases such as tuberculosis, leishmaniasis, Hansen's disease, atypical mycobacteriosis, hepatitis B and C, HIV, HTLV-1, mononucleosis, Chagas disease, Yersinia. However, the relationship between citrullination and viral infections is not entirely clear since it is still a relatively recent area of research and requires further investigation.

In a paper by Pratesi and colleagues[42], it is hypothesized that the deletion of a viral sequence of the Epstein-Barr virus is able to generate epitopes that are recognized by anti-filaggrin antibodies (AFAs). AFAs only react with citrullinated filaggrin, both in vivo and in vitro. A comparison of the sequences most frequently recognized by AFAs demonstrated that they have a higher affinity towards citrulline molecules flanked by neutral amino acids such as glycine, serine or threonine. Similar amino acid repeats have also been found in proteins that bind nucleic acids and, among these, some are of viral origin (for example the proteins that regulate the transcription of herpesviruses). One of the nuclear proteins encoded by EBV, the Epstein-Barr nuclear antigen 1 (EBNA-1), contains in the N-terminal region a sequence (35 amino acids -58) characterized by six glycine-arginine repeats. The data obtained by Pratesi and colleagues indicate that the antibodies present in the sera of patients with RA react with the sequence 35-58 of the EBNA-1 deiminated protein, encoded by EBV. These results suggest that EBV infection plays a role in the induction of specific antibodies in RA[42]. Furthermore, anti-viral citrullinated peptide (VCP) antibodies have been observed to recognize deiminated EBNA-

1 in vivo by ionomycin treatment of infected lymphoblast cells. Unfortunately, in this study it was not checked whether the infection with EBV alone was able to independently induce the activation of PAD enzymes. In a subsequent work carried out by the same research group, the authors wanted to analyze the frequency of antibodies (anti-VCP) in the sera of patients with RA through a peptide derived from EBV, in which the arginine residues are replaced by residues of citrulline. Anti-VPC antibodies were found in 45% of sera from RA patients versus less than 5% in controls. In addition, the level of positivity for these antibodies correlates positively with the level of anti-CCP: this indicates that VCP can be considered a potential substrate for detecting ACPA[43].

Moreover, a correlation has recently emerged between PADs dysregulation and viral infections. Casanova et all found that the antiviral activity of the LL37 protein is compromised by human rhinovirus (HRV) induced citrullination. In their study show that citrullination of LL-37 reduced its antiviral activity directed against HRV[44]. Furthermore, while the anti-rhinovirus activity of LL-37 results in dampened epithelial cell inflammatory responses, citrullination of the peptide, and a loss in antiviral activity, ameliorates this effect. Their study also demonstrates that HRV infection upregulates PAD2 protein expression and increases levels of protein citrullination, including histone H3, in human bronchial epithelial cells. In conclusion, they hypothesize that increased PADI gene expression and HDP citrullination during infection may represent a novel viral evasion mechanism.

1.6 PADs inhibitors

Over the years, the presence of citrullinated proteins and antibodies to citrullinated proteins has been correlated with an increasingly large number of pathological pictures, this has created the need for drugs that block the citrullination process by acting on the responsible enzymes.

The first bioactive PAD inhibitor was unearthed in 2006, when Thompson and colleagues described the synthesis and characterization of F-amidine[45]. The structure of this molecule is based on benzoylarginine starch (BAA), which is one of the best small molecule substrates identified for PADs. Since the PADs preferred to hydrolyze a positively charged guanidine residue, it was hypothesized that the replacement of one of the amino groups with a methylene fluoride would have generated a compound capable of covalently modifying the enzyme.

The resulting compound containing fluoroacetamidine retains the positive charge and the ability to form most of the hydrogen bonds of guanidine, but due to the nature of the strongly electrophilic fluorine, it can react with a residue of cysteine present in the active site of the enzyme.

This compound, F-amidine, inhibits PAD4 with a kinact / KI value of 3000 M-1s-1. Further experiments have shown that F-amidine irreversibly inhibits the enzyme by modifying Cys645 and is bioavailable.
Building on the success of F-amidine, a series of compounds were synthesized in which the nature of the positively charged head varied, replacing fluorine with a chlorine or hydrogen atom. We also tried to define the optimal length of the side chain by synthesizing compounds in which this chain ranged from two to four methylene units. Among these compounds, Cl-amidine was found to be the most potent inhibitor of PAD with a kinact / KI value of 13,000 M-1s-1 for PAD4[45]. It was subsequently shown that it also inhibits other PAD isozymes with similar potency[46]. Cl-amidine is structurally identical to F-amidine except that it has a chlorine atom instead of fluorine.
Compounds without an electron-holding group or with longer side chains were, in contrast, rather weak inhibitors of PAD.
Cl-amidine, like F-amidine, preferentially inhibits the active, calcium-bound form of the enzyme. This is due to the fact that the binding of calcium to the enzyme promotes a conformational change that moves Cys645 in an optimal position to promote substrate catalysis and therefore also the binding with the inhibitor.
Both compounds irreversibly inhibit the enzyme through nucleophilic attack on the amide carbon. This leads to the formation of a stabilized tetrahedral intermediate through the donation of protons by His471, as generally occurs in the reaction catalyzed by PADs. The sulfur of Cys645 attacks the halogenated carbon to form a three-membered sulphonic ring, the rupture of this ring forms the stable adduct of the thioether, irreversibly modifying the active site of the enzyme.
In order to obtain PAD inhibitors with improved cellular bioavailability, Thompson and colleagues synthesized BB-Cl-amidine[22]. This compound retains the essential elements of Cl-amidine (i.e. the reactive chloroacetamidine head and side chain length), but possesses a C-terminal benzimidazole, designed to limit the proteolysis of C-terminal starch. Furthermore, the N-terminal benzoyl group is replaced by a biphenyl, which has been incorporated to increase the hydrophobicity of the molecule and thus facilitate its cellular uptake (Fig. 1.9). In vitro, Cl-amidine and BB-Cl-amidine have similar potency and selectivity, however, on cell cultures the potency of BB-Cl-amidine increases more than 20-fold. This increase is likely due to increased absorption as Cl-amidine and BB-Cl-amidine have been observed to have similar hepatic clearance properties. Furthermore, studying the pharmacokinetics of BB-Cl-amidine it was shown that it has a significantly longer in vivo half-life than Cl-amidine (1.75 h vs ~ 15 min).
Multiple studies have tested these inhibitors as drugs in the various pathologies in which aberrant activation of PAD is involved. For example, Cl-amidine and BB-Cl-amidine inhibition of PADs has

been shown to reduce NET formation and protect against lupus-related vascular damage in the New Zealand mixed lupus model (MRL / lpr mice)[47].

Since the five isoforms of PAD are differentially expressed in tissues in physiological and pathological conditions, selective inhibitors for the different isoforms would be required to specifically study their biological role.

Thompson and colleagues recently attempted to develop a selective inhibitor for PAD2, which plays a key role in the onset and progression of multiple sclerosis, rheumatoid arthritis and breast cancer. To obtain this compound, a series of benzoimidazole derivatives of Cl-amidine have been synthesized. The replacement of the N-terminal and the C-terminal result in an increase in power and selectivity for PAD2 of over one hundred times[33]. Such a compound will be fundamental to elucidate the biological roles of this isozyme and may be useful for the treatment of specific diseases in which PAD2 activity is altered.

2. Herpesviruses

2.1 The Herpesvirus Family

Herpesviruses comprise a large group of highly prevalent and widely distributed pathogens that infect humans and other vertebrates. They are generally characterized by a large double stranded DNA genome, strict host specificity, and the ability to establish latency and life-long persistence with spontaneous reactivation periods[48]. Within the Herpesviridae family, the viruses are divided into three subfamilies on the basis of their genome sequences: alpha-, beta- and gammaherpesvirinae, respectively. At least eight prominent herpesviruses possess an ability to successfully infect humans: herpes simplex viruses 1 and 2 (HSV-1 and -2), varicella-zoster virus (VZV), human cytomegalovirus (HCMV), human herpesviruses 6 and 7, Epstein-Barr virus (EBV), and Kaposi's sarcoma-associated herpesvirus (KSHV)[49].

Herpesviruses are extremely common within the human population, as about 90% of humans appear seropositive at least for one or even two herpesviruses simultaneously[50]. Generally, herpesvirus infection runs asymptomatic in immunocompetent hosts, whereas in immunocompromised hosts infection results in multiple disorders, including oral and genital herpes, infectious mononucleosis, conjunctivitis, and encephalitis[48], and have been linked to neurodegenerative diseases[51–53] and several malignancies, such as Kaposi’s sarcoma and numerous EBV-associated tumors[54–58]. Left untreated, severe infections may result in a fatal outcome[59–61]. The major problem within disease management and prevention is the fact that effective treatment and/or preventive therapies are currently

unavailable. Global burden of herpesvirus diseases has been steadily increasing over the last decades[62–64] and therefore should remain a high priority for targeted antiviral drug research, vaccine development, and clinical management of patients.

Human herpesviruses are ancient viruses that have been evolving within the human population for millions of years[49,65,66] and it is highly likely that the process of their evolution is still ongoing[67]. Herpesviruses across all three subfamilies (alpha-, beta-, and gammaherpesvirinae) have been following the phylogenetic history of the hosts, which can be tracked by multitudes of within-host viral lineage duplications[68]. Moreover, the host-specific nature of herpesviruses and their ability to establish dormant life-long infections indicate that they have co-evolved with their humans exquisitely well. The great adaptation to the host reflects their ability to persist "unseen" by the immune system and thus resist eradication from the infected organism. At the same time, selective pressure exerted by the immune system prompts herpesviruses to develop multiple evasion strategies, serving as further proof for virus-host adaptation upon their long coevolutionary path. Unsurprisingly, herpesviruses possess an arsenal of elaborate strategies to avoid or counteract immune responses of the host[69]. Among them human cytomegalovirus, acts as a true master of immune evasion[70,71].

During my PhD years, we focused on two members of herpesvirus family: human cytomegalovirus (HCMV) and Human herpes simplex virus 1 (HSV1), a prototype of betaherpesvirus and alphaherpesvirus, respectively.

2.1.1 Virion structure

Herpesviruses have historically been defined based on the architecture of the virion. A typical herpesvirion consists of a core containing a linear double-stranded DNA (dsDNA, ranging from 124–295 kb in length)[49,67]; an icosahedral capsid approximately 125 nm in diameter containing 161 capsomeres with a hole running down their long axis, plus one capsomeric structure that serves as the portal for packaging and release of the viral genome (the complex of the core and capsid is the nucleocapsid)[72,73]; an amorphous-appearing, sometimes asymmetric material that surrounds the nucleocapsid and is designated the tegument; and an envelope containing viral glycoprotein spikes on its surface[74,75].

2.1.2 Genome organization

Herpesvirus DNAs extracted from virions and characterized to date are linear and double stranded, but they circularize immediately on release from nucleocapsids into the nuclei of infected cells[49].

Distinguishing features of herpesvirus DNAs include their length and base composition. The length of herpesvirus DNAs varies from approximately 124 to 295 kbp (specifically 230 kbp for HCMV and 150 kbp for HSV)[67,76]. The variability in genome lengths of different herpesviruses is distinct from the generally less extensive polymorphism in the size of DNAs of individual viruses. Thus, herpesvirus genomes contain terminal and internal reiterated sequences that can vary in copy number, as well as sequences that can be lost or duplicated during passage in cell culture, leading to intraspecies variation in genome lengths that can exceed 10 kbp. The base composition of herpesvirus DNAs varies from 31% to 77% total G+C content[76,77].

Based on their sequence arrangement the Herpesvirus genomes can be divided into six groups designated by the letters A to F. In the viral genomes of HSV and HCMV, both belonging to group E, the sequences of both terminals are repeated with an inverted orientation and juxtaposed internally, dividing the genomes into two components, each of which consists of unique sequences flanked by unrelated pairs of inverted repetitions. In this case, both components can reverse with respect to each other and the DNA extracted from infected virions or cells consists of four equimolar populations of isomers that differ in the relative orientation of the two components[67,78,79].

2.1.3 Viral replication and antiviral target

Herpesvirus entry is a highly complex process involving numerous viral and cellular factors[80]. Viral attachment to the host cell surface is the first step for viral entry, followed by interactions between multiple viral glycoproteins and binding receptors to facilitate capsid penetration. Each subfamily of herpesviruses has its own specific receptor-binding glycoproteins and receptors[81]. For HSV, gD is the required receptor-binding protein, which binds to Nectin-1, herpesvirus entry mediator (HVEM), and a modified form of heparan sulfate receptors, which triggers membrane fusion together with heterodimers (gH-gL) and gB while VZV lacks gD. EBV gp42 binding to human leukocyte antigen (HLA) class II is required to enter B cells, but only gH-gL and gB are sufficient for epithelial cell entry[82]. HCMV requires gH-gL together with gO in trimeric complexes before cell entry into fibroblast, epithelial and endothelial cells[83]. All herpesviruses require gH-gL as an essential component of their fusion steps of entry[81]. Then the nucleocapsid travels along microtubules to the nuclear membrane, where viral DNA is released for replication in the nucleus. Once in the nucleus, viral DNA is transcribed into mRNA by cellular RNA polymerase II.

Herpesviruses are strictly regulated in three temporal cascades: immediateearly (IE), early (E), and late (L) gene expression during their productive cycles[84]. IE genes encode regulatory proteins, which are transcribed immediately by host RNA polymerase II to take control of cell defense and activate

E genes. These encode necessary proteins for viral DNA replication, and the L genes mostly encode viral structural proteins. After all mRNAs are transcribed in the nucleus and translated into proteins in the cytoplasm, capsid proteins and viral DNA are packed to form new virions. Mature virions are released by exocytosis. Herpesviruses may follow similar pathways, except some proceed at slower paces than others[48].

Herpetic viral infection can be treated in many ways. Antiviral agents with inhibitory and virucidal effects including interference of viral adsorption, inhibition of the viral penetration into cells, and inhibition of viral biosynthesis and release are used to treat the infection[85]. Moreover, immunomodulators are used to boost the host immune system and induce autophagy against the virus. Antiviral drugs aim to interfere with one of the stages in any virus replication cycle, such as inhibiting DNA polymerase, reverse transcriptase, neuraminidase, and other mechanisms of actions targeted by broad-spectrum antiviral drugs[86]. The most common therapeutic drugs used in clinical practice are nucleoside analogs, which affect the DNA replication process. Acyclovir, penciclovir, valacyclovir, famciclovir, ganciclovir, and other related prodrugs are highly effective in actively replicating viruses against herpesvirus infection[87]. However, in immunocompromised patients, prolonged treatment with these drugs is more likely to develop drug-resistant strains due to mutations of thymidine nucleoside kinase or DNA polymerase. Recently, there have been some live-attenuated vaccines available for boosting cell-mediated immunity to reduce the incidence of varicella-zoster viral recurrence, but rare side effects are increasingly described due to the increased administration of VZV live vaccines worldwide[88]. No effective herpesvirus vaccine has been found to completely eradicate viral infection due to the occurrence of reactivation until now. Hence, the development of alternatively new antiviral agents with minimal side effects and reduced toxicity plays a significant role in targeting different mechanisms rather than nucleoside analogues and vaccines.

2.2 HCMV

2.2.1 The Human Cytomegalovirus

Human cytomegalovirus is an important clinical pathogen around the globe, with higher seroprevalence in countries with lower socioeconomic status[89,90]. Like the other herpesviruses, HCMV maintains asymptomatic infection in the immunocompetent population, but leads to severe complications and even elevated mortality among hosts whose immune system is either weakened or immature[91–93]. Thus, the risk groups for HCMV infection include solid organ or stem cell transplant recipients treated with immunosuppressors[94,95] and cancer and AIDS patients[93,96], who commonly

display symptoms such as gastrointestinal ulceration, hepatitis, pneumonitis or retinitis, which can lead to blindness[97].

Neonates with immature immune systems are one of the major target groups for HCMV infection, resulting in severe congenital disease[98]. HCMV is a leading cause of congenital disease among newborns worldwide due to an infectious agent[99] and the disease prevalence is higher than Down syndrome, spina bifida or fetal alcohol syndrome[89,100]. The outcome of congenital HCMV varies from patient to patient, from birth defects to permanent neurological morbidities, such as hearing and vision loss, microcephaly, cerebral palsy, and long-term intellectual disability[98,101]. Furthermore, mounting evidence suggests that HCMV may contribute to immunosenescence in the elderly[102–105] and is linked to a number of autoimmune[106,107], inflammatory and vascular diseases[108–110], as well as some cancers[111–117].

A significant challenge in combating HCMV infection is the absence of a vaccine or antiviral treatment[118,119]. Commonly, in addition to immunoglobulin from seropositive individual, ganciclovir and its oral analog valganciclovir are used as antiviral agents that target viral polymerase (pUL54) or viral phosphotransferase (pUL97)[120], despite their significant toxicity, which limits their administration to some categories of patients, such as pregnant women[120,121]. Other antiviral drugs, foscarnet and cidofovir, are listed as alternatives for adults with severe HCMV infection, but not children due to their toxicity and side effects, such as renal neutropenia.

Besides, the failings of current antiviral agents are not limited to their high toxicity. As there is an increased resistance among treated patients due to occurring mutations in UL97 and UL54[122,123]. Numerous mutations associated with antiviral resistance were identified through sequencing of both genes, reported by multiple groups upon studying different patient cohorts[124–126]. While frequencies of these resistant mutants among transplant recipients vary, they generally range between 5%–10%[127]. Finally, the latest proposed antiviral drugs include maribavir and letermovir, however, their use is currently limited and not yet universally available[128].

Although numerous HCMV vaccine candidates have been tested previously, including live attenuated vaccines AD169 and Towne[118], Towne/Toledo chimeric viruses, DNA vectors, vaccines based on dense bodies[129] or glycoprotein subunits, eventually they have all failed to provide an efficient antibody response and progress further in clinical trials[130].

The live attenuated vaccines proved insufficiently immunogenic due to substantial genetic differences between commonly used attenuated laboratory strains (e.g. AD169, Towne) and clinical isolates that directly impact viral tropism, in particular, its ability to infect endo- and epithelial cells[131]. Given the fact that HCMV displays a strong tendency toward interstrain polymorphism, it is not surprising that HCMV antigenic differences may affect neutralizing host antibody responses. Furthermore,

immunogenicity testing of vaccine candidatesis complicated by the absence of an HCMV animal model. Nevertheless, the search for an HCMV vaccine currently continues through the engagement of multiple strategies which currently demonstrate some potential[130]. For instance, a few AD169 based candidates, such as attenuated vaccine or genetically engineered replicationdefective AD169-derivate, contain restored pentameric complexes gH/gL/pUL128-131 and aim at improving potency of neutralizing antibodies[132].

Other experimental vaccine candidates, such as subunit protein[133], DNA vectored[134], and viral vectored vaccines[135–137], are currently being assessed at different phases of clinical trials. Lately, the common idea prevails that an efficient vaccine candidate should contain multiple epitopes and thus be able to trigger both humoral and cellular responses to reach its maximum efficacy. For instance, a multi-antigen HCMV vaccine that combines co-expressed pp65 and gB, has been tested upon congenital infection in a guinea pig model[138].

Taking into account all the above-mentioned reasons, undoubtedly, there is an existent need for better prognostic markers for HCMV disease and more accurate patient risk stratification, as well as improved individualized therapeutics based on new targets and with limited toxicity and efficient vaccine.

2.2.2 HCMV and autoimmune disease

A large body of evidence has shown how HCMV can use several of its genes to manipulate the innate and adaptive immune system of the infected subject[70,106,139,140]. This feature alongside many others, such as its wide tropism[141–143], its ability to persist in the host during phases of latency and reactivation and, as already mentioned, its global distribution[90], makes HCMV a candidate etiological agent of AD. Even though a causative link between HCMV infection and AD may appear difficult to determine epidemiologically given the widespread prevalence of HCMV and the rare occurrence of AD, mounting evidence has increasingly associated HCMV infection with rheumatologic diseases—e.g., systemic lupus erythematosus (SLE), systemic sclerosis (SSc), rheumatoid arthritis (RA)—and neurological disorders—e.g., multiple sclerosis (MS), and enteric disorders.

Despite the great effort, researchers have not yet been able to discriminate whether HCMV is an initiator of AD or just an epiphenomenon that may just simply exacerbate the course of AD. In this regard, multiple mechanisms have been proposed to explain HCMV-induced autoimmunity. Indeed, HCMV can induce or perpetuate autoimmunity through different processes that can be divided into two categories: 1) antigen-specific (i.e., molecular mimicry); and 2) non-antigen-specific (i.e., bystander activation). From an immunopathological perspective, HCMV can trigger or sustain

autoimmunity through the following three mechanisms: *i)* autoantibodies production; *ii)* enhanced inflammation; and *iii)* vascular damage[144].

Through a mechanism defined as “molecular mimicry”, viral epitopes that are highly similar to host determinants may induce the development of antibodies that attack the self at the level of specific tissues, as it has been hypothesized for the viral tegument protein pp65 in SLE patients[145]. Intriguingly, upon HCMV infection immunocompetent hosts tend to develop an autoimmune reaction through the generation of autoantibodies, which occurs more frequently in those individuals with a systemic involvement[146]. Furthermore, HCMV-infected bone marrow transplant recipients quite often develop organ-specific autoantibodies against the human aminopeptidase CD13[147] or common phospholipid[148], whereas solid organ transplant recipients develop non-organ-specific autoantibodies[149]. Accordingly, hypergammaglobulinemia, cryoglobulinemia and autoantibody production are common features of HCMV-driven mononucleosis[150,151]. This unspecific hyperactivation of humoral immunity is thought to represent a mechanism of viral immune evasion because it curbs host B-cell responses. Once the tissue is infected, activated antigen-presenting cells (APCs) are attracted to the infection site and release high levels of cytokines and chemokines that activate autoreactive T or B cells, leading to loss of tolerance, a phenomenon called “bystander activation”. Finally, several pieces of evidence suggest a role of HCMV infection in vascular damage and stenosis, an event that is quite frequent and fatal in patients with AD[152,153].

There is also some evidence indicating that HCMV infection and AD mutually affect each other. In particular, while primary or secondary HCMV infection can induce chronic, systemic type I inflammation, which may promote autoimmunity, eventually leading to AD[154], autoimmune flares can also trigger HCMV reactivation[154]. Intriguingly, HCMV-induced immunosuppression, which has severe consequences in transplant recipients, may also play a protective role in the course of AD[155].

2.3 HSV

2.3.1 The Herpes Simplex Virus

Of the Herpes Simplex virus, two different serotypes called HSV-1 and HSV-2 are known, which possess common antigens and specific antigens[48,156]. The two serotypes have a sequence homology equal to 83% in the protein coding regions, they also have a similar genome structure and a similar replication cycle. They have a similar tissue tropism: they cause lytic infections in epithelial cells and fibroblasts, persistent infections in lymphocytes and macrophages, and finally they give latent infections in neurons. They manifest themselves with lesions characterized by vesicular elements

gathered in clusters that regress spontaneously. The vesicles are filled with serum and contain large amounts of actively proliferating viruses[157–159].

HSV-1 is mainly responsible for the herpetic manifestations of the skin or mucous membranes located mainly in the skin in the perioral area (herpes labialis) or in the buccal mucosa (herpetic gingivostomatitis). It is often contracted in early childhood from human to human contagion directed by injury bearers[160]. HSV-2 is responsible for genital herpes localized on the skin and genital mucous membranes (genital herpes). Primary infection can be neonatal, but more often it is contracted through sexual contact and is typical of adulthood[161,162].

Primary HSV infection is caused by inoculation of the virus through a cutaneous or mucosal entrance. Locally, the large number of virions generated by viral replication, due to a direct cytopathic effect, leads to structural changes and ultimately death of the host cells. Furthermore, the virus infects the sensory endings of a neuron, with transport of the nucleocapsid by retrograde axonal way to the body of the neuron, in the corresponding sensory ganglion. The immune system eventually controls the local infection, but the virus survives in the 'sanctuary' of the sensory ganglion[163,164].

The virus can reactivate, resuming a replicative cycle following exposure to UV rays, stress, hyperthermia, hormonal treatment. In this case, it follows the axonal path in a centrifugal way, in the direction of the mucus cutaneous territory corresponding to the first infection where there is a new lytic cycle, with clinical manifestations generally less intense than the first infection.

It may happen that the secondary manifestation does not perfectly match the primary, as the neuron in which it goes into latency can innervate a larger region of skin[54,165].

2.3.2 HSV1 and Alzheimer

As anticipated in the previous chapter, Herpes simplex virus type 1 (HSV-1) is a neurotropic virus that establishes a latent infection in sensory, typically the trigeminal, ganglia and periodically reactivates giving rise to the well-known cold sores and blisters on orolabial mucosa[48,166]. Following reactivation, HSV-1 may also reach the central nervous system (CNS)[167–169] resulting in either a severe, but rare, form of herpetic encephalitis (HSE) or establishing latency[170,171].

Increasing evidence suggests a possible link between HSV-1 infection and Alzheimer's disease (AD), the most common neurodegenerative disease associated with dementia in the elderly[172–175]. Previous studies demonstrated the presence of HSV-1 genome in the brain of AD patients, particularly those carrying the ε4 allele of apolipoprotein E that is a risk factor for AD[176,177]. More recently, population-based studies correlated the level and avidity index of anti-HSV-1 IgG and IgM (markers of HSV-1 infection and reactivation, respectively) to the risk of developing AD[164,178–180]. Many in vitro studies

supported the hypothesis that HSV-1 is involved in AD pathogenesis. Specifically, it was demonstrated that HSV-1 infection in cultured neurons induces the amyloidogenic processing of amyloid precursor protein (APP) and intra- and extra-neuronal accumulation of amyloid-β protein (Aβ) and other neurotoxic APP fragments[181]. It was also shown that HSV-1 activates intracellular processes leading to neurodegeneration through different mechanisms, most of them driven by APP fragments[182–184].

Others studies pointed out the effects of HSV-1 infection on the microtubule-associated protein tau, showing that the virus triggers its hyperphosphorylation and aggregation, likely leading to its deposition in neurofibrillary tangles (NFTs), another hallmark of AD[185,186].

Altogether these data strongly support the hypothesis that accumulation of AD biomarkers triggered by recurrent infection, if not properly cleared, may drive neurodegeneration. This view was supported by the results of in vivo studies showing inflammatory and neurodegenerative markers in a mouse model of HSE induced by intranasal HSV-1 inoculation[187]. However, a clear cause-effect relationship among multiple HSV-1 reactivations, active viral replication in the brain, accumulation of AD molecular markers and cognitive deficits has yet to be demonstrated.

3. Coronavirus

3.1 The coronaviridae family

Emerging zoonotic RNA viruses have repeatedly attracted the attention of researchers and the world over the past few decades. Among them, coronaviruses (CoVs) need special attention due to the high mortality rates, the lack of effective therapies and the high rate of transmissibility that allows them to trigger epidemics that can quickly cross geographical borders. Furthermore, large numbers of highly diverse CoVs have been identified in animal hosts, especially in bat species, from which they may have the potential to spread to other species giving rise to future epidemics [188].

The Coronaviridae family consists of enveloped viruses with a single-stranded positive-sense RNA genome which primarily infect mammals and birds and includes four genera: alphacoronavirus, betacoronavirus, gammacoronavirus and deltacoronavirus. To date, seven human coronaviruses are known which fall into the alpha and betacoronavirus genera[189]. Human coronaviruses HCoV-229E and HCoV-OC43 were the first to be identified (1966 and 1967 respectively) and together with the more recently emerged HCoV-NL63 (2004) and HCoV-HKU1 (2005) generally cause mild disease of the upper respiratory tract and together they are responsible for about 10-30% of common colds [190,191]. However, in the past two decades, spillover events from wildlife have given rise to three highly

pathogenic coronaviruses that can cause severe respiratory disease in humans. Severe acute respiratory syndrome coronavirus (SARS-CoV) emerged in 2002[192,193], nine years later the Middle East respiratory syndrome coronavirus (MERS-CoV) appeared in 2011 [79,194], finally, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in 2019 [195,196]. All three of these highly pathogenic coronaviruses belong to the betacoronavirus genera. SARS-CoV2 is the causal agent of the ongoing outbreak of atypical pneumonia (COVID-19) and the World Health Organization declared it a "Public Health Emergency of International Interest" on January 30, 2020 (www.who.int). The current epidemic, along with the real possibility that new coronaviruses could continue to emerge from the wide wildlife reservoir, highlights the urgent need to develop broad-spectrum therapeutic agents to address the current SARS-CoV2 outbreak and manage possible future epidemics.

3.2 Antiviral drugs

Most of the approved antiviral drugs are the so-called direct-acting antiviral agents (DAAs), compounds designed against viral proteins deemed essential for infection. For example, remdesivir, and molnupiravir, a new oral antiviral highly effective in preventing severe disease based on the results of a recent Phase 2a trial[197], are nucleoside analogue prodrugs acting as competitive substrates for virally-encoded RNA-dependent RNA polymerase (RdRp)[132,198]. The most important antiviral drugs tested to treated Coronavirus infection are detailed below.

Lopinavir/Ritonavir (LPV/r). Lopinavir is a protease inhibitor used for the treatment of Human Immunodefciency Virus (HIV) infection. Lopinavir binds HIV protease and prevents the Gag/Pol polyprotein cleavage with the consequent production of immature non-infectious viral particles. Ritonavir enhances lopinavir plasmatic concentration by inhibiting cytochrome P450 3A isoform. The drug demonstrated inhibitory activity against SARS-CoV and MERS-CoV in vitro and in animal models. Moreover, a 2004 open-label study showed that the association LPV/r with ribavirin compared to ribavirin alone had a positive efect on ARDS development and mortality in patients with SARS-CoV severe pneumonia[199]. On this basis, LPV/r has been tested since the beginning of the pandemic. In the single available randomized controlled clinical trial LPV/r therapy has not proven effectiveness in patients with severe disease and was not superior to standard therapy when started 12 days or later after symptoms onset. In the few studies evaluating LPN/r in association with other drugs, such as Umifenovir, Ribavirin and Interferons, combination therapies seem to perform better than LPN/r alone[199,200]. Further studies with a better study design on larger populations are urgently needed to establish if LPN/r could be an effective therapeutic option for COVID-19 disease and to evaluate the appropriate timing of administration of this drug.

Remdesivir. Remdesivir is an adenosine analogue, which is included in nascent viral RNA chains resulting in premature termination. Formerly evaluated for the treatment of Ebola Virus infection, Remdesivir is a promising broad-spectrum antiviral drug active against a wide range of RNA viruses, including SARS-CoV and MERS-CoV in cultured cells, mice and non-human primate models. The drug seems to reduce viral load in lung tissue in SARS-CoV pneumonia in mice, leading to improvement in ventilatory function and healing of the damaged tissue[201]. It is worthy to acknowledge, however, that when the drug was administered after peak viral replication with airway epithelium damage already occurred, it did not improve survival and healing. This indicates that Remdesivir should be administered at the early stages of the disease[202]. Since SARS-CoV and SARS-CoV2 RNA-dependent RNA polymerase (RdRp) share 96% sequence identity, it could be hypothesized that drugs targeting viral RdRp proteins of SARS-CoV can be effective on SARS-CoV2 too[203]. Indeed, Wang et al. demonstrated that, compared to other antiviral drugs, Remdesivir contrasts and controls SARS-CoV2 infection in vitro at lower micromolar concentrations with a very high selectivity index[204]. The Food and Drug Administration has authorized Remdesivir compassionate use for the treatment of adults and children with severe COVID-19 disease who do not respond to other treatments[205]. A case series of 61 patients treated with Remdesivir seemed to show drug effectiveness by improving oxygen support class and symptoms even when administered later after symptoms onset (9–12 days) in severe disease[206]. However, when tested in larger populations, the results seemed to be more conflicting. In a randomized double-blind trial, no differences were noted in 28 days mortality and clinical improvement in patients treated with Remdesivir compared with subjects treated with placebo; it should be acknowledged that this study was performed on patients with advanced disease and didn't reach its enrolment target[203]. Conversely, a larger study on a population of 1059 hospitalized COVID-19 patients demonstrated that Remdesivir started at an advanced disease stage was superior compared to standard therapy in time to recovery and mortality; however, albeit statistically significant, the results of Remdesivir were only slightly better than placebo[206]. Finally, a study that compared 5-day versus 10-day Remdesivir administration failed to demonstrate better clinical outcomes depending on Remdesivir therapy duration; moreover, a higher burden of side effects was observed among patients in the 10-day therapy group[207]. Since all the studies testing Remdesivir in COVID-19 disease up to now are based on patients with severe disease in an advanced stage, it would be useful to evaluate the drug activity on moderate COVID-19 patients, administering the molecule soon after symptoms onset to observe if, in these categories of subjects, better results and an higher efficacy could be reached.

Favipiravir. Favipiravir is a new generation RdRp inhibitor active against a wide range of viruses. Its use, which is hampered by signifcant side effects (teratogenicity and suicide induction), is authorized

in Japan, but not in Europe and USA, for infuenza treatment when other antivirals are not effective[208]. Several studies with Favipiravir among COVID-19 patients have been authorized by regulatory agencies, many of which are still ongoing. The available clinical trials are based on small populations, are not randomized and not double-blinded. Moreover, groups features were heterogeneous, making it hard to draw conclusions on its effect on COVID-19 disease. In the two studies reported in Table 1—Favipiravir section, it seemed slightly more effective than LPN/r and Umifenovir in improving imaging, viral clearance and clinical signs[208,209].

Umifenovir. Umifenovir is a small indole-derived molecule developed in Russia and approved in Russia and China for prophylaxis and treatment of influenza and other respiratory viral infections. It is able to block viral fusion with the cell membrane in Infuenza A and B viruses. Umifenovir and its derivate Umifenovir mesylate had been reported to have antiviral activity against SARS-CoV in cell cultures[210]. A retrospective study based on a small population of patients afected by moderate and severe COVID-19 disease failed to observe signifcant differences in viral clearance and symptoms resolution between Umifenovir and standard therapy[211]. The limited evidence regarding the use of this drug on COVID-19 patients makes it impossible to draw conclusions.

REFERENCES

1. György, B., Tóth, E., Tarcsa, E., Falus, A. & Buzás, E. I. Citrullination: A posttranslational modification in health and disease. *Int. J. Biochem. Cell Biol.* **38**, 1662–1677 (2006).

2. Orgován, G. & Noszál, B. The complete microspeciation of arginine and citrulline. *J. Pharm. Biomed. Anal.* **54**, 965–971 (2011).

3. Vossenaar, E. R., Zendman, A. J. W., Van Venrooij, W. J. & Pruijn, G. J. M. PAD, a growing family of citrullinating enzymes: Genes, features and involvement in disease. *BioEssays* **25**, 1106–1118 (2003).

4. Totaro, M. C. *et al.* Porphyromonas gingivalis and the pathogenesis of rheumatoid arthritis: Analysis of various compartments including the synovial tissue. *Arthritis Res. Ther.* **15**, (2013).

5. Liao, F. *et al.* Porphyromonas gingivalis may play an important role in the pathogenesis of periodontitis-associated rheumatoid arthritis. *Med. Hypotheses* **72**, 732–735 (2009).

6. Witalisom, E., Thompson, R. & Hofseth, L. Protein Arginine Deiminases and Associated Cirtullination. *Anu. Music. Rev. Musicol. del CSIC* **16**, 199–227 (2002).

7. Mondal, S. & Thompson, P. R. Protein Arginine Deiminases (PADs): Biochemistry and Chemical Biology of Protein Citrullination. *Acc. Chem. Res.* **52**, 818–832 (2019).

8. Senshu, T., Akiyama, K., Ishigami, A. & Nomura, K. Studies on specificity of peptidylarginine deiminase reactions using an immunochemical probe that recognizes an enzymatically deiminated partial sequence of mouse keratin K1. *J. Dermatol. Sci.* **21**, 113–126 (1999).

9. Zhang, X. *et al.* Peptidylarginine deiminase 1-catalyzed histone citrullination is essential for early embryo development OPEN. (2016) doi:10.1038/srep38727.

10. Feroz, S., Muhammad, N., Ranayake, J. & Dias, G. Keratin - Based materials for biomedical applications. *Bioact. Mater.* **5**, 496–509 (2020).

11. Terakawa, H., Takahara, H. & Sugawara, K. *Three Types of Mouse Peptidylarginine Deiminase: Characterization and Tissue Distribution1*. *J. Biochem* vol. 110 (1991).

12. Ishigami, A. & Maruyama, N. Importance of research on peptidylarginine deiminase and citrullinated proteins in age-related disease. *Geriatr. Gerontol. Int.* **10**, 53–58 (2010).

13. Musse, A. A. *et al.* Peptidylarginine deiminase 2 (PAD2) overexpression in transgenic mice leads to myelin loss in the central nervous system. *DMM Dis. Model. Mech.* **1**, 229–240 (2008).

14. Kanno, T. *et al.* Human peptidylarginine deiminase type III: Molecular cloning and nucleotide sequence of the cDNA, properties of the recombinant enzyme, and immunohistochemical localization in human skin. *J. Invest. Dermatol.* **115**, 813–823 (2000).

15. Nachat, R. *et al.* Peptidylarginine deiminase isoforms are differentially expressed in the anagen hair follicles and other human skin appendages. *J. Invest. Dermatol.* **125**, 34–41 (2005).

16. Jones, J. E., Causey, C. P., Knuckley, B., Slack-Noyes, J. L. & Thompson, P. R. Protein

arginine deiminase 4 (PAD4): Current understanding and future therapeutic potential. *Current Opinion in Drug Discovery and Development* vol. 12 616–627 (2009).

17. Chang, X. *et al.* Increased PADI4 expression in blood and tissues of patients with malignant tumors. (2009) doi:10.1186/1471-2407-9-40.

18. Chang, X. & Han, J. Expression of Peptidylarginine Deiminase Type 4 (PAD4) in Various Tumors. doi:10.1002/mc.20169.

19. Esposito, G. *et al.* Peptidylarginine deiminase (PAD) 6 is essential for oocyte cytoskeletal sheet formation and female fertility. *Mol. Cell. Endocrinol.* **273**, 25–31 (2007).

20. Kan, R. *et al.* Regulation of mouse oocyte microtubule and organelle dynamics by PADI6 and the cytoplasmic lattices. *Dev. Biol.* **350**, 311–322 (2011).

21. Ishigami, A. *et al.* Mass spectrometric identification of citrullination sites and immunohistochemical detection of citrullinated glial fibrillary acidic protein in Alzheimer's disease brains. *J. Neurosci. Res.* **93**, 1664–1674 (2015).

22. Knight, J. S. *et al.* Peptidylarginine deiminase inhibition disrupts NET formation and protects against kidney, skin and vascular disease in lupus-prone MRL/ *lpr* mice. *Ann. Rheum. Dis.* **74**, 2199–2206 (2015).

23. Acharya, N. K. *et al.* Neuronal PAD4 expression and protein citrullination: Possible role in production of autoantibodies associated with neurodegenerative disease. *J. Autoimmun.* **38**, 369–380 (2012).

24. Valesini, G. *et al.* Citrullination and autoimmunity. *Autoimmun. Rev.* **14**, 490–497 (2015).

25. van Venrooij, W. J., van Beers, J. J. B. C. & Pruijn, G. J. M. Anti-CCP antibodies: the past, the present and the future. *Nat. Rev. Rheumatol.* **7**, 391–398 (2011).

26. Silman, A. J. & Pearson, J. E. Epidemiology and genetics of rheumatoid arthritis. *Arthritis Res.* **4**, S265–S272 (2002).

27. Makrygiannakis, D. *et al.* Citrullination is an inflammation-dependent process. *Ann. Rheum. Dis.* **65**, 1219–1222 (2006).

28. Mohamed, B. M. *et al.* Citrullination as early-stage indicator of cell response to Single-Walled Carbon Nanotubes. *Sci. Rep.* **3**, 24–26 (2013).

29. Yuzhalin, A. E. Citrullination in Cancer. *Cancer Res.* **79**, 1274–1284 (2019).

30. Biron, B. M. *et al.* E-Mail Cl-Amidine Prevents Histone 3 Citrullination and Neutrophil Extracellular Trap Formation, and Improves Survival in a Murine Sepsis Model. (2016) doi:10.1159/000448808.

31. Arisan, E. D., Uysal-Onganer, P. & Lange, S. Putative roles for peptidylarginine deiminases in COVID-19. *Int. J. Mol. Sci.* **21**, 1–29 (2020).

32. Cherrington, B. D. *et al.* Potential role for PAD2 in gene regulation in breast cancer cells. *PLoS One* **7**, 1–12 (2012).

33. Muth, A. *et al.* Development of a Selective Inhibitor of Protein Arginine Deiminase 2. *J. Med. Chem.* **60**, 3198–3211 (2017).

34. Lange, S. *et al.* Peptidylarginine deiminases—roles in cancer and neurodegeneration and possible avenues for therapeutic intervention via modulation of exosome and microvesicle

(EMV) release? *Int. J. Mol. Sci.* **18**, 1–20 (2017).

35. Bradford, C. M. *et al.* Localisation of citrullinated proteins in normal appearing white matter and lesions in the central nervous system in multiple sclerosis. *J. Neuroimmunol.* **273**, 85–95 (2014).

36. Schirmer, L., Srivastava, R. & Hemmer, B. To look for a needle in a haystack: The search for autoantibodies in multiple sclerosis. *Mult. Scler. J.* **20**, 271–279 (2014).

37. Papuć, E., Kurys-Denis, E., Krupski, W., Tatara, M. & Rejdak, K. Can antibodies against glial derived antigens be early biomarkers of hippocampal demyelination and memory loss in Alzheimer's disease? *J. Alzheimer's Dis.* **48**, 115–121 (2015).

38. Acharya, N. K. *et al.* Neuronal PAD4 expression and protein citrullination: Possible role in production of autoantibodies associated with neurodegenerative disease. *J. Autoimmun.* **38**, 369–380 (2012).

39. Masutomi, H. *et al.* Induction of peptidylarginine deiminase 2 and 3 by dibutyryl cAMP via cAMP-PKA signaling in human astrocytoma U-251MG cells. *J. Neurosci. Res.* **95**, 1503–1512 (2017).

40. Ishigami, A. *et al.* Abnormal accumulation of citrullinated proteins catalyzed by peptidylarginine deiminase in hippocampal extracts from patients with Alzheimer's disease. *J. Neurosci. Res.* **80**, 120–128 (2005).

41. U, K. P., Subramanian, V., Nicholas, A. P., Thompson, P. R. & Ferretti, P. Modulation of calcium-induced cell death in human neural stem cells by the novel peptidylarginine deiminase-AIF pathway. *Biochim. Biophys. Acta - Mol. Cell Res.* **1843**, 1162–1171 (2014).

42. Pratesi, F., Tommasi, C., Anzilotti, C., Chimenti, D. & Migliorini, P. Deiminated Epstein-Barr virus nuclear antigen 1 is a target of anti-citrullinated protein antibodies in rheumatoid arthritis. *Arthritis Rheum.* **54**, 733–741 (2006).

43. Pratesi, F. *et al.* Antibodies to a new viral citrullinated peptide, VCP2: fine specificity and correlation with anti-cyclic citrullinated peptide (CCP) and anti-VCP1 antibodies. *Clin. Exp. Immunol.* **164**, 337–345 (2011).

44. Casanova, V. *et al.* Citrullination Alters the Antiviral and Immunomodulatory Activities of the Human Cathelicidin LL-37 During Rhinovirus Infection. *Front. Immunol.* **11**, (2020).

45. Yuan, L. *et al.* Inhibitors and Inactivators of Protein Arginine Deiminase 4: Functional and Structural Characterization. *Biochemistry* **45**, 11727–11736 (2006).

46. Causey, C. P. *et al.* The development of o-F-amidine and o-Cl-amidine as second generation PAD inhibitors. *J Med Chem* **54**, 6919–6935 (2011).

47. Ledet, M. M. *et al.* BB-Cl-Amidine as a novel therapeutic for canine and feline mammary cancer via activation of the endoplasmic reticulum stress pathway. *BMC Cancer* **18**, 1–13 (2018).

48. Arvin, A. *et al.* Human Herpesviruses. *Hum. Herpesviruses Biol. Ther. Immunoprophyl.* 10–26 (2007).

49. McGeoch, D. J., Rixon, F. J. & Davison, A. J. Topics in herpesvirus genomics and evolution. *Virus Res.* **117**, 90–104 (2006).

50. Boppana, S. B. & Fowler, K. B. Persistence in the population: epidemiology and transmisson. *Hum. Herpesviruses Biol. Ther. Immunoprophyl.* 795–813 (2007)

doi:10.1017/CBO9780511545313.045.

51. Harris, S. A. & Harris, E. A. Molecular mechanisms for herpes simplex virus type 1 pathogenesis in Alzheimer's disease. *Front. Aging Neurosci.* **10**, 1–24 (2018).

52. Koyuncu, O. O., Hogue, I. B. & Enquist, L. W. Virus infections in the nervous system. *Cell Host Microbe* **13**, 379–393 (2013).

53. Duarte, L. F. *et al.* Herpes simplex virus type 1 infection of the central nervous system: Insights into proposed interrelationships with neurodegenerative disorders. *Front. Cell. Neurosci.* **13**, 1–23 (2019).

54. Preston, C. M. & Efstathiou, S. Molecular basis of HSV latency and reactivation. *Hum. Herpesviruses Biol. Ther. Immunoprophyl.* (2007).

55. Dillon, P. J. & Damania, B. Human kaposi's sarcoma-associated herpesvirus: Molecular biology and oncogenesis. *Hum. Oncog. Viruses* **289**, 269–316 (2009).

56. Cesarman, E. Gammaherpesvirus and lymphoproliferative disorders in immunocompromised patients. *Cancer Lett.* **305**, 163–174 (2011).

57. Jha, H. C., Banerjee, S. & Robertson, E. S. The role of gammaherpesviruses in cancer pathogenesis. *Pathogens* **5**, (2016).

58. Taylor, G. S., Long, H. M., Brooks, J. M., Rickinson, A. B. & Hislop, A. D. The immunology of epstein-barr virus-induced disease. *Annu. Rev. Immunol.* **33**, 787–821 (2015).

59. Jereb, M., Lainscak, M., Marin, J. & Popovic, M. Herpes simplex virus infection limited to the brainstem. *Wien. Klin. Wochenschr.* **117**, 495–499 (2005).

60. Corey, L. & Wald, A. Maternal and Neonatal HSV Infections. *N. Engl. J. Med.* **361**, 1376–1385 (2009).

61. Jouan, Y. *et al.* Long-term outcome of severe herpes simplex encephalitis: A population-based observational study. *Crit. Care* **19**, 1–9 (2015).

62. Khan, G. & Hashim, M. J. Global burden of deaths from epstein-barr virus attributable malignancies 1990-2010. *Infect. Agent. Cancer* **9**, (2014).

63. Navarro, D. Expanding role of cytomegalovirus as a human pathogen. *J. Med. Virol.* **88**, 1103–1112 (2016).

64. Tzellos, S. & Farrell, P. J. Epstein-barr virus sequence variation-biology and disease. *Pathogens* **1**, 156–175 (2012).

65. McGeoch, D. J., Dolan, A. & Ralph, A. C. Toward a Comprehensive Phylogeny for Mammalian and Avian Herpesviruses. *J. Virol.* **74**, 10401–10406 (2000).

66. McGeoch, D. J., Cook, S., Dolan, A., Jamieson, F. E. & Telford, E. A. R. Molecular phylogeny and evolutionary timescale for the family of mammalian herpesviruses. *J. Mol. Biol.* **247**, 443–458 (1995).

67. Sharma, V., Mobeen, F. & Prakash, T. Comparative Genomics of Herpesviridae Family to Look for Potential Signatures of Human Infecting Strains. *Int. J. Genomics* **2016**, (2016).

68. Kitchen, A., Shackelton, L. A. & Holmes, E. C. Family level phylogenies reveal modes of macroevolution in RNA viruses. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 238–243 (2011).

69. Stempel, M., Chan, B. & Brinkmann, M. M. Coevolution pays off: Herpesviruses have the

license to escape the DNA sensing pathway. *Med. Microbiol. Immunol.* **208**, 495–512 (2019).

70. McSharry, B. P., Avdic, S. & Slobedman, B. Human cytomegalovirus encoded homologs of cytokines, chemokines and their receptors: Roles in immunomodulation. *Viruses* **4**, 2448–2470 (2012).

71. Galitska, G. *et al.* Catch me if you can: the arms race between human cytomegalovirus and the innate immune system. *https://doi.org/10.2217/fvl-2018-0189* **14**, 247–263 (2019).

72. Taylor, L. F. & Yuan, Y. Structure of Herpesvirus Capsid Sheds Light on Drug Discovery. *Trends Microbiol.* **26**, 391–392 (2018).

73. Herpesvirales. *Virus Taxon.* 99–107 (2012) doi:10.1016/B978-0-12-384684-6.00005-7.

74. Burke, H. G. & Heldwein, E. E. Crystal Structure of the Human Cytomegalovirus Glycoprotein B. *PLoS Pathog.* **11**, 1–21 (2015).

75. Schauflinger, M., Villinger, C., Mertens, T., Walther, P. & von Einem, J. Analysis of human cytomegalovirus secondary envelopment by advanced electron microscopy. *Cell. Microbiol.* **15**, 305–314 (2013).

76. Murphy, E. & Shenk, T. E. Human cytomegalovirus genome. *Curr. Top. Microbiol. Immunol.* **325**, 1–19 (2008).

77. Novotny, J., Rigoutsos, I., Coleman, D. & Shenk, T. In silico structural and functional analysis of the human cytomegalovirus (HHV5) genome. *J. Mol. Biol.* **310**, 1151–1166 (2001).

78. Deiss, L. P., Chou, J. & Frenkel, N. Domains Sequence. **59**, 605–618 (1986).

79. Zaki, A. M., van Boheemen, S., Bestebroer, T. M., Osterhaus, A. D. M. E. & Fouchier, R. A. M. Isolation of a Novel Coronavirus from a Man with Pneumonia in Saudi Arabia. *N. Engl. J. Med.* **367**, 1814–1820 (2012).

80. Agelidis, A. M. & Shukla, D. Cell entry mechanisms of HSV: What we have learned in recent years. *Future Virol.* **10**, 1145–1154 (2015).

81. Connolly, S. A., Jardetzky, T. S. & Longnecker, R. The structural basis of herpesvirus entry. *Nat. Rev. Microbiol.* **19**, 110–121 (2021).

82. Sathiyamoorthy, K. *et al.* Structural basis for Epstein-Barr virus host cell tropism mediated by gp42 and gHgL entry glycoproteins. *Nat. Commun.* **7**, (2016).

83. Zhou, M., Lanchy, J.-M. & Ryckman, B. J. Human Cytomegalovirus gH/gL/gO Promotes the Fusion Step of Entry into All Cell Types, whereas gH/gL/UL128-131 Broadens Virus Tropism through a Distinct Mechanism. *J. Virol.* **89**, 8999–9009 (2015).

84. Gruffat, H., Marchione, R. & Manet, E. Herpesvirus late gene expression: A viral-specific pre-initiation complex is key. *Front. Microbiol.* **7**, 1–15 (2016).

85. Li, W. *et al.* Traditional chinese medicine as a potential source for hsv-1 therapy by acting on virus or the susceptibility of host. *Int. J. Mol. Sci.* **19**, 1–23 (2018).

86. Teuton, J. R. & Brandt, C. R. Sialic Acid on Herpes Simplex Virus Type 1 Envelope Glycoproteins Is Required for Efficient Infection of Cells. *J. Virol.* **81**, 3731–3739 (2007).

87. Field, H. J. & Anthony Vere Hodge, R. Recent developments in anti-herpesvirus drugs. *Br.*

Med. Bull. **106**, 213–249 (2013).

88. Andrei, G. & Snoeck, R. Advances and perspectives in the management of varicella-zoster virus infections. *Molecules* **26**, (2021).

89. Cannon, M. J., Schmid, D. S. & Hyde, T. B. Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. *Rev. Med. Virol.* **20**, 202–213 (2010).

90. Zuhair, M. *et al.* Estimation of the worldwide seroprevalence of cytomegalovirus: A systematic review and meta-analysis. *Rev. Med. Virol.* **29**, 1–6 (2019).

91. Söderberg-Nauclér, C. Cytomegalovirus in human brain tumors: Role in pathogenesis and potential treatment options. *World J. Exp. Med.* **5**, 1 (2015).

92. Stern, L. *et al.* Human cytomegalovirus latency and reactivation in allogeneic hematopoietic stem cell transplant recipients. *Front. Microbiol.* **10**, 1–13 (2019).

93. Griffiths, P., Baraniak, I. & Reeves, M. The pathogenesis of human cytomegalovirus. *J. Pathol.* **235**, 288–297 (2015).

94. Sagedal, S. *et al.* A prospective study of the natural course of cytomegalovirus infection and disease in renal allograft recipients. *Transplantation* **70**, 1166–1174 (2000).

95. Ljungman, P., Hakki, M. & Boeckh, M. Cytomegalovirus in Hematopoietic Stem Cell Transplant Recipients. *Hematol. Oncol. Clin. North Am.* **25**, 151–169 (2011).

96. Wohl, D. A. *et al.* Antiretroviral Therapies. **38**, 538–544 (2005).

97. Fisher, S., Genbacev, O., Maidji, E. & Pereira, L. Human Cytomegalovirus Infection of Placental Cytotrophoblasts In Vitro and In Utero: Implications for Transmission and Pathogenesis. *J. Virol.* **74**, 6808–6820 (2000).

98. Britt, W. J. Congenital Human Cytomegalovirus Immunity. *J. Virol.* **91**, 1–7 (2017).

99. Manicklal, S., Emery, V. C., Lazzarotto, T., Boppana, S. B. & Gupta, R. K. The 'Silent' global burden of congenital cytomegalovirus. *Clin. Microbiol. Rev.* **26**, 86–102 (2013).

100. Cannon, M. J. Congenital cytomegalovirus (CMV) epidemiology and awareness. *J. Clin. Virol.* **46**, 6–10 (2009).

101. Dollard, S. C., Grosse, S. D. & Ross, D. S. New estimates of the prevalence of neurological and sensory sequelae and mortality associated with congenital cytomegalovirus infection. *Rev. Med. Virol.* **17**, 355–363 (2007).

102. Pawelec, G. Hallmarks of human 'immunosenescence' : Adaptation or dysregulation? *Immun. Ageing* **9**, 1 (2012).

103. Pawelec, G. Immunosenenescence: Role of cytomegalovirus. *Exp. Gerontol.* **54**, 1–5 (2014).

104. Wistuba-Hamprecht, K., Frasca, D., Blomberg, B., Pawelec, G. & Derhovanessian, E. Age-associated alterations in γδ T-cells are present predominantly in individuals infected with Cytomegalovirus. *Immun. Ageing* **10**, 1 (2013).

105. Tu, W. & Rao, S. Mechanisms underlying T cell immunosenescence: Aging and cytomegalovirus infection. *Front. Microbiol.* **7**, 1–12 (2016).

106. Halenius, A. & Hengel, H. Human cytomegalovirus and autoimmune disease. *Biomed Res. Int.* **2014**, (2014).

107. Marou, E. *et al.* Increased immunoreactivity against human cytomegalovirus UL83 in systemic sclerosis. *Clin. Exp. Rheumatol.* **35**, S31–S34 (2017).

108. Lin, W. R., Wozniak, M. A., Wilcock, G. K. & Itzhaki, R. F. Cytomegalovirus is present in a very high proportion of brains from vascular dementia patients. *Neurobiol. Dis.* **9**, 82–87 (2002).

109. Ji, Y. N., An, L., Zhan, P. & Chen, X. H. Cytomegalovirus infection and coronary heart disease risk: A meta-analysis. *Mol. Biol. Rep.* **39**, 6537–6546 (2012).

110. Du, Y., Zhang, G. & Liu, Z. Human cytomegalovirus infection and coronary heart disease: A systematic review. *Virology Journal* vol. 15 (2018).

111. Michaelis, M., Doerr, H. W. & Cinatl, J. The story of human cytomegalovirus and cancer: Increasing evidence and open questions. *Neoplasia* **11**, 1–9 (2009).

112. Melnick, M., Sedghizadeh, P. P., Allen, C. M. & Jaskoll, T. Human cytomegalovirus and mucoepidermoid carcinoma of salivary glands: Cell-specific localization of active viral and oncogenic signaling proteins is confirmatory of a causal relationship. *Exp. Mol. Pathol.* **92**, 118–125 (2012).

113. Taher, C. *et al.* High prevalence of human cytomegalovirus in brain metastases of patients with primary breast and colorectal cancers. *Transl. Oncol.* **7**, 732–740 (2014).

114. Price, R. L. *et al.* Human Cytomegalovirus is Present in Alveolar Soft Part Sarcoma. *Appl. Immunohistochem. Mol. Morphol.* **25**, 615–619 (2017).

115. Rådestad, A. F. *et al.* Impact of Human Cytomegalovirus Infection and its Immune Response on Survival of Patients with Ovarian Cancer. *Transl. Oncol.* **11**, 1292–1300 (2018).

116. Herbein, G. The human cytomegalovirus, from oncomodulation to oncogenesis. *Viruses* **10**, 1–16 (2018).

117. Cobbs, C. Cytomegalovirus is a tumor-associated virus: armed and dangerous. *Curr. Opin. Virol.* **39**, 49–59 (2019).

118. Schleiss, M. R., Permar, S. R. & Plotkin, S. A. Progress toward development of a vaccine against congenital cytomegalovirus infection. *Clin. Vaccine Immunol.* **24**, 1–20 (2017).

119. Plotkin, S. A. & Boppana, S. B. Vaccination against the human cytomegalovirus. *Vaccine* **37**, 7437–7442 (2019).

120. Britt, W. J. & Prichard, M. N. New therapies for human cytomegalovirus infections. *Antiviral Res.* **159**, 153–174 (2018).

121. Benoist, G. *et al.* Management of pregnancies with confirmed cytomegalovirus fetal infection. *Fetal Diagn. Ther.* **33**, 203–214 (2013).

122. Piret, J. & Boivin, G. Clinical development of letermovir and maribavir: Overview of human cytomegalovirus drug resistance. *Antiviral Res.* **163**, 91–105 (2019).

123. Minces, L. R. *et al.* Ganciclovir-Resistant cytomegalovirus infections among lung transplant recipients are associated with poor outcomes despite treatment with foscarnet-containing regimens. *Antimicrob. Agents Chemother.* **58**, 128–135 (2014).

124. Lurain, N. S. & Chou, S. Antiviral drug resistance of human cytomegalovirus. *Clin. Microbiol. Rev.* **23**, 689–712 (2010).

125. Chou, S. Rapid in vitro evolution of human cytomegalovirus UL56 mutations that confer letermovir resistance. *Antimicrob. Agents Chemother.* **59**, 6588–6593 (2015).

126. Cytomegalovirus, R. H., Piret, J., Goyette, N. & Boivin, G. crossm Drug Susceptibility and Replicative Capacity of Multidrug-Resistant Genes. **61**, 54–57 (2017).

127. Gilbert, C. & Boivin, G. Human cytomegalovirus resistance to antiviral drugs. *Antimicrob. Agents Chemother.* **49**, 873–883 (2005).

128. Van Leer Buter, C. C. *et al.* Antiviral-resistant cytomegalovirus infections in solid organ transplantation in the Netherlands. *J. Antimicrob. Chemother.* **74**, 2370–2376 (2019).

129. Cayatte, C. *et al.* Cytomegalovirus Vaccine Strain Towne-Derived Dense Bodies Induce Broad Cellular Immune Responses and Neutralizing Antibodies That Prevent Infection of Fibroblasts and Epithelial Cells. *J. Virol.* **87**, 11107–11120 (2013).

130. Xia, L., Su, R., An, Z., Fu, T. M. & Luo, W. Human cytomegalovirus vaccine development: Immune responses to look into vaccine strategy. *Hum. Vaccines Immunother.* **14**, 292–303 (2018).

131. Gerna, G., Percivalle, E., Sarasini, A., Baldanti, F. & Revello, M. G. The attenuated Towne strain of human cytomegalovirus may revert to both endothelial cell tropism and leuko- (neutrophil- and monocyte-) tropism in vitro. *J. Gen. Virol.* **83**, 1993–2000 (2002).

132. Warren, T. K. *et al.* Therapeutic efficacy of the small molecule GS-5734 against Ebola virus in rhesus monkeys. *Nature* **531**, 381–385 (2016).

133. Griffiths, P. D. *et al.* Cytomegalovirus glycoprotein-B vaccine with MF59 adjuvant in transplant recipients: A phase 2 randomised placebo-controlled trial. *Lancet* **377**, 1256–1263 (2011).

134. Kharfan-Dabaja, M. A. *et al.* A novel therapeutic cytomegalovirus DNA vaccine in allogeneic haemopoietic stem-cell transplantation: A randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet Infect. Dis.* **12**, 290–299 (2012).

135. Reap, E. A. *et al.* Development and preclinical evaluation of an alphavirus replicon particle vaccine for cytomegalovirus. *Vaccine* **25**, 7441–7449 (2007).

136. Loomis, R. J. *et al.* Vectored co-delivery of human cytomegalovirus gH and gL proteins elicits potent complement-independent neutralizing antibodies. *Vaccine* **31**, 919–926 (2013).

137. Liu, J., Jaijyan, D. K., Tang, Q. & Zhu, H. Promising cytomegalovirus-based vaccine vector induces robust CD8+ T-cell response. *Int. J. Mol. Sci.* **20**, (2019).

138. Schleiss, M. R. *et al.* Additive protection against congenital cytomegalovirus conferred by combined glycoprotein B/pp65 vaccination using a lymphocytic choriomeningitis virus vector. *Clin. Vaccine Immunol.* **24**, 1–16 (2017).

139. Dunn, W. *et al.* Functional profiling of a human cytomegalovirus genome. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 14223–14228 (2003).

140. Gugliesi, F. *et al.* Where do we stand after decades of studying human cytomegalovirus? *Microorganisms* vol. 8 (2020).

141. Sinzger, C. *et al.* Modification of human cytomegalovirus tropism through propagation in vitro is associated with changes in the viral genome. *J. Gen. Virol.* **80**, 2867–2877 (1999).

142. Nguyen, C. C. & Kamil, J. P. Pathogen at the gates: Human cytomegalovirus entry and cell

tropism. *Viruses* **10**, (2018).

143. Gerna, G., Kabanova, A. & Lilleri, D. Human cytomegalovirus cell tropism and host cell receptors. *Vaccines* **7**, 1–18 (2019).

144. Gugliesi, F. *et al.* Human cytomegalovirus and autoimmune diseases: Where are we? *Viruses* vol. 13 (2021).

145. HoHsieh, A. *et al.* B cell epitope of human cytomegalovirus phosphoprotein 65 (HCMV pp65) induced anti-dsDNA antibody in BALB/c mice. *Arthritis Res. Ther.* **19**, 1–14 (2017).

146. Varani, S. *et al.* Generalized Wegener's granulomatosis in an immunocompetent adult after cytomegalovirus mononucleosis and bacterial urinary tract infection. *Arthritis Rheum.* **60**, 1558–1562 (2009).

147. Giugni, T. D. *et al.* Neutralization of human cytomegalovirus by human CD13-specific antibodies. *J. Infect. Dis.* **173**, 1062–1071 (1996).

148. Mengarelli, A. *et al.* High levels of antiphospholipid antibodies are associated with cytomegalovirus infection in unrelated bone marrow and cord blood allogeneic stem cell transplantation. *Br. J. Haematol.* **108**, 126–131 (2000).

149. Toyoda, M. *et al.* Cytomegalovirus infection induces anti-endothelial cell antibodies in cardiac and renal allograft recipients. *Transpl. Immunol.* **5**, 104–111 (1997).

150. Wager, O., Räsänen, J. A., Hagman, A. & Klemola, E. Mixed Cryoimmunoglobulinaemia in Infectious Mononucleosis and Cytomegalovirus Mononucleosis. *Int. Arch. Allergy Immunol.* **34**, 345–361 (1968).

151. Kantor, G. L., Goldberg, L. S., Johnson, B. L., Derechin, M. M. & Barnett, E. V. Immunologic abnormalities induced by postperfusion cytomegalovirus infection. *Ann. Intern. Med.* **73**, 553–558 (1970).

152. Lunardi, C. *et al.* Antibodies against human cytomegalovirus in the pathogenesis of systemic sclerosis: A gene array approach. *PLoS Med.* **3**, 94–108 (2006).

153. Streblow, D. N., Orloff, S. L. & Nelson, J. A. Acceleration of allograft failure by cytomegalovirus. *Curr. Opin. Immunol.* **19**, 577–582 (2007).

154. Van De Berg, P. J. *et al.* Human cytomegalovirus induces systemic immune activation characterized by a type 1 cytokine signature. *J. Infect. Dis.* **202**, 690–699 (2010).

155. George, M. J. *et al.* The Independent Role of Cytomegalovirus as a Risk Factor for Invasive Fungal Disease in Orthotopic Liver Transplant Recipients. *Am. J. Med.* **103**, 106–113 (1997).

156. Jeffery-Smith, A. & Riddell, A. Herpesviruses. *Med. (United Kingdom)* **49**, 780–784 (1996).

157. Rechenchoski, D. Z., Faccin-Galhardi, L. C., Linhares, R. E. C. & Nozawa, C. Herpesvirus: an underestimated virus. *Folia Microbiol. (Praha).* **62**, 151–156 (2017).

158. Chang, E., Galle, L., Maggs, D., Estes, D. M. & Mitchell, W. J. Pathogenesis of Herpes Simplex Virus Type 1-Induced Corneal Inflammation in Perforin-Deficient Mice. *J. Virol.* **74**, 11832–11840 (2000).

159. Thellman, N. M. & Triezenberg, S. J. Herpes simplex virus establishment, maintenance, and reactivation: In vitro modeling of latency. *Pathogens* **6**, (2017).

160. Saleh, D., Yarrarapu, S. N. S. & Sharma, S. Herpes Simplex Type 1. *StatPearls* (2021).

161. Wald, A. Genital herpes. *West. J. Med.* **170**, 343–347 (1999).

162. Jaishankar, D. & Shukla, D. Genital herpes: Insights into sexually transmitted infectious disease. *Microb. Cell* **3**, 438–450 (2016).

163. Verzosa, A. L. *et al.* Herpes Simplex Virus 1 Infection of Neuronal and Non-Neuronal Cells Elicits Specific Innate Immune Responses and Immune Evasion Mechanisms. *Front. Immunol.* **12**, 1–17 (2021).

164. Kobayashi, N. *et al.* Increase in the IgG avidity index due to herpes simplex virus type 1 reactivation and its relationship with cognitive function in amnestic mild cognitive impairment and Alzheimer's disease. *Biochem. Biophys. Res. Commun.* **430**, 907–911 (2013).

165. Chen, S.-H. *et al.* Efficient Reactivation of Latent Herpes Simplex Virus from Mouse Central Nervous System Tissues. *J. Virol.* **80**, 12387–12392 (2006).

166. Roizman, B. *et al.* Herpesviridae. *Intervirology* **16**, 201–217 (1981).

167. Kastrukoff, L. *et al.* Central nervous system infection and immune response in mice inoculated into the lip with herpes simplex virus type 1. *J. Neuroimmunol.* **2**, 295–305 (1982).

168. Schmutzhard, E. Viral infections of the CNS with special emphasis on herpes simplex infections. *J. Neurol.* **248**, 469–477 (2001).

169. Lewandowski, G., Zimmerman, M. N., Denk, L. L., Porter, D. D. & Prince, G. A. Herpes simplex type 1 infects and establishes latency in the brain and trigeminal ganglia during primary infection of the lip in cotton rats and mice. *Arch. Virol.* **147**, 167–179 (2002).

170. Sekizawa, T. & Openshaw, H. Encephalitis resulting from reactivation of latent herpes simplex virus in mice. *J. Virol.* **50**, 263–266 (1984).

171. Stahl, J. P. & Mailles, A. Herpes simplex virus encephalitis update. *Curr. Opin. Infect. Dis.* **32**, 239–243 (2019).

172. De Chiara, G. *et al.* Infectious agents and neurodegeneration. *Mol. Neurobiol.* **46**, 614–638 (2012).

173. Piacentini, R. *et al.* HSV-1 and Alzheimer's disease: More than a hypothesis. *Front. Pharmacol.* **5 MAY**, 1–9 (2014).

174. Itzhaki, R. F. Overwhelming evidence for a major role for herpes simplex virus type 1 (Hsv1) in alzheimer's disease (ad); underwhelming evidence against. *Vaccines* **9**, 1–19 (2021).

175. Marcocci, M. E. *et al.* Herpes Simplex Virus-1 in the Brain: The Dark Side of a Sneaky Infection. *Trends Microbiol.* **28**, 808–820 (2020).

176. Itzhaki, R. F. *et al.* Herpes simplex virus type 1 in brain and risk of Alzheimer's disease. *Lancet* **349**, 241–244 (1997).

177. Itzhaki, R. F. Herpes simplex virus type 1 and Alzheimer's disease: possible mechanisms and signposts. *FASEB J.* **31**, 3216–3226 (2017).

178. Letenneur, L. *et al.* Seropositivity to Herpes Simplex Virus antibodies and risk of Alzheimer's disease: A population-based cohort study. *PLoS One* **3**, 1–5 (2008).

179. Mancuso, R. *et al.* Titers of Herpes Simplex Virus Type 1 antibodies positively correlate

with grey matter volumes in Alzheimer's disease. *J. Alzheimer's Dis.* **38**, 741–745 (2014).

180. Lövheim, H., Gilthorpe, J., Adolfsson, R., Nilsson, L. G. & Elgh, F. Reactivated herpes simplex infection increases the risk of Alzheimer's disease. *Alzheimer's Dement.* **11**, 593–599 (2015).

181. De Chiara, G. *et al.* APP processing induced by herpes simplex virus type 1 (HSV-1) yields several APP fragments in human and rat neuronal cells. *PLoS One* **5**, (2010).

182. Civitelli, L. *et al.* Herpes simplex virus type 1 infection in neurons leads to production and nuclear localization of APP intracellular domain (AICD): implications for Alzheimer's disease pathogenesis. *J. Neurovirol.* **21**, 480–490 (2015).

183. Piacentini, R. *et al.* Herpes Simplex Virus type-1 infection induces synaptic dysfunction in cultured cortical neurons via GSK-3 activation and intraneuronal amyloid-β protein accumulation. *Sci. Rep.* **5**, 1–14 (2015).

184. De Chiara, G. *et al.* Herpes simplex virus-type1 (HSV-1) impairs DNA repair in cortical neurons. *Front. Aging Neurosci.* **8**, 1–12 (2016).

185. Zambrano, Á. *et al.* Neuronal cytoskeletal dynamic modification and neurodegeneration induced by infection with herpes simplex virus type 1. *J. Alzheimer's Dis.* **14**, 259–269 (2008).

186. Wozniak, M. A., Frost, A. L. & Itzhaki, R. F. Alzheimer's disease-specific tau phosphorylation is induced by herpes simplex virus type 1. *J. Alzheimer's Dis.* **16**, 341–350 (2009).

187. Martin, C. *et al.* Inflammatory and neurodegeneration markers during asymptomatic HSV-1 reactivation. *J. Alzheimer's Dis.* **39**, 849–859 (2014).

188. Fan, Y., Zhao, K., Shi, Z. L. & Zhou, P. Bat coronaviruses in China. *Viruses* vol. 11 (2019).

189. Su, S. *et al.* Epidemiology, Genetic Recombination, and Pathogenesis of Coronaviruses. (2016) doi:10.1016/j.tim.2016.03.003.

190. Leao, J. C. *et al.* Coronaviridae—Old friends, new enemy! *Oral Dis.* 0–3 (2020) doi:10.1111/odi.13447.

191. Paules, C. I., Marston, H. D. & Fauci, A. S. Coronavirus Infections-More Than Just the Common Cold. *JAMA - Journal of the American Medical Association* vol. 323 707–708 (2020).

192. Weiss, S. R. & Navas-Martin, S. Coronavirus Pathogenesis and the Emerging Pathogen Severe Acute Respiratory Syndrome Coronavirus. *Microbiol. Mol. Biol. Rev.* **69**, 635–664 (2005).

193. Ksiazek, T. G. *et al.* A Novel Coronavirus Associated with Severe Acute Respiratory Syndrome. *N. Engl. J. Med.* **348**, 1953–1966 (2003).

194. De Wit, E., Van Doremalen, N., Falzarano, D. & Munster, V. J. SARS and MERS: Recent insights into emerging coronaviruses. *Nature Reviews Microbiology* vol. 14 523–534 (2016).

195. Wu, F. *et al.* A new coronavirus associated with human respiratory disease in China. *Nature* **579**, 265–269 (2020).

196. Lu, R. *et al.* Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet* **395**, 565–574 (2020).

197. Fischer, W. *et al.* Molnupiravir, an Oral Antiviral Treatment for COVID-19. *medRxiv Prepr. Serv. Heal. Sci.* (2021) doi:10.1101/2021.06.17.21258639.

198. Beigel, J. H. *et al.* Remdesivir for the Treatment of Covid-19 — Final Report. *N. Engl. J. Med.* **383**, 1813–1826 (2020).

199. Cao, B. *et al.* A Trial of Lopinavir–Ritonavir in Adults Hospitalized with Severe Covid-19. *N. Engl. J. Med.* **382**, 1787–1799 (2020).

200. Deng, L. *et al.* Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID- 19 . The COVID-19 resource centre is hosted on Elsevier Connect , the company ' s public news and information . (2020).

201. Wang, Y. *et al.* Remdesivir in adults with severe COVID-19: a randomised, double-blind, placebo-controlled, multicentre trial. *Lancet* **395**, 1569–1578 (2020).

202. Sheahan, T. P. *et al.* Broad-spectrum antiviral GS-5734 inhibits both epidemic and zoonotic coronaviruses. *Sci. Transl. Med.* **9**, (2017).

203. Agostini, M. L. *et al.* Coronavirus susceptibility to the antiviral remdesivir (GS-5734) is mediated by the viral polymerase and the proofreading exoribonuclease. *MBio* **9**, 1–15 (2018).

204. Wang, M. *et al.* Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. *Cell Res.* **30**, 269–271 (2020).

205. Grein, J. *et al.* Compassionate Use of Remdesivir for Patients with Severe Covid-19. *N. Engl. J. Med.* **382**, 2327–2336 (2020).

206. Beigel, J. H. *et al.* Remdesivir for the Treatment of Covid-19 — Final Report. *N. Engl. J. Med.* **383**, 1813–1826 (2020).

207. Goldman, J. D. *et al.* Remdesivir for 5 or 10 Days in Patients with Severe Covid-19. *N. Engl. J. Med.* **383**, 1827–1837 (2020).

208. Chen, C. *et al.* Favipiravir versus Arbidol for COVID-19: A randomized clinical trial. *medRxiv* (2020) doi:10.1101/2020.03.17.20037432.

209. Liu, K., Chen, Y., Lin, R. & Han, K. Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID- 19 . The COVID-19 resource centre is hosted on Elsevier Connect , the company ' s public news and information. *J. Infect.* **80**, 14–18 (2020).

210. Blaising, J., Polyak, S. J. & Pécheur, E. Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID- 19 . The COVID-19 resource centre is hosted on Elsevier Connect , the company ' s public news and information . (2020).

211. Lian, N. *et al.* Umifenovir treatment is not associated with improved outcomes in patients with coronavirus disease 2019: a retrospective study. *Clin. Microbiol. Infect.* **26**, 917–921 (2020).

PUBLICATIONS

1. Citrullination and Herpes Simplex Virus type 1 infection: implications for neurodegenerative disorders. *Selina Pasquero, Francesca Gugliesi, Matteo Biolatti, Gloria Griffante, Valentina dell'oste, Camilla Albano, Greta Bajetto, Marco De Andrea.*
Manuscript in preparation.

2. Novel antiviral activity of PAD inhibitors against human beta-coronaviruses HCoV-OC43 and SARS-CoV-2. *Selina Pasquero, Francesca Gugliesi, Gloria Griffante, Valentina Dell'Oste, Matteo Biolatti, Camilla Albano, Greta Bajetto, Serena Delbue, Lucia Signorini, Maria Dolci, Santo Landolfo, Marco De Andrea.*
Antiviral Research, Under Revision.

3. Human Cytomegalovirus-Induced Host Protein Citrullination Is Crucial for Viral Replication. *Griffante Gloria, Francesca Gugliesi, Selina Pasquero, Valentina Dell'Oste, Matteo Biolatti, Ari J. Salinger, and others*.
Nature Communications, 12.1 (2021).

4. Human Cytomegalovirus and Autoimmune Diseases: Where Are We? *Gugliesi Francesca, Selina Pasquero, Gloria Griffante, Sara Scutera, Camilla Albano, Sergio Fernando Castillo Pacheco, and others.*
Viruses (MDPI AG, 2021).

5. Where Do We Stand after Decades of Studying Human Cytomegalovirus? *Gugliesi Francesca, Alessandra Coscia, Gloria Griffante, Ganna Galitska, Selina Pasquero, Camilla Albano, and others.* Microorganisms (MDPI AG, 2020).

6. Tuning the Orchestra: HCMV vs. Innate Immunity. *Dell'Oste Valentina, Matteo Biolatti, Ganna Galitska, Gloria Griffante, Francesca Gugliesi, Selina Pasquero, and others.*
Frontiers in Microbiology (Frontiers Media S.A., 2020).

Manuscript in Preparation

Citrullination and Herpes Simplex Virus type 1 infection: implications for neurodegenerative disorders.

SELINA PASQUERO (1), GLORIA GRIFFANTE (3), FRANCESCA GUGLIESI (1), MATTEO BIOLATTI (1), CAMILLA ALBANO (1), SERGIO FERNANDO CASTILLO PACHECO (1), MARCO DE ANDREA (1, 2), VALENTINA DELL'OSTE (1), SANTO LANDOLFO (1).

(1) Department of Public Health and Pediatric Sciences, University of Turin, Turin, Turin, Italy
(2) Center for Translational Research on Autoimmune and Allergic Disease-CAAD, 28100 Novara, Italy.
(3) Department of Translational Medicine, University of Eastern Piedmont, Novara 28100, Italy.

Abstract

Herpes simplex virus type 1 (HSV-1) is a neurotropic virus that infects most humans, attaining 90% prevalence by the sixth decade of life. The virus remains latent in neuronal cell bodies and reactivates due to stress, illness and other unknown factors throughout an individual's life. The disease appears as cold sores, typically seen on the lips or face, with primary infection usually during childhood. In some cases, individuals can develop adverse reactions such as herpes simplex encephalitis (HSE) and recent evidence suggests the involvement of HSV-1 in the etiology of Alzheimer's disease (AD). Sporadic AD is a complex multifactorial neurodegenerative disease with evidence indicating coexisting multi-pathogen and inflammatory etiologies. Citrullination is a post-translational modification (PTM) catalyzed by peptidyl arginine deiminases (PAD) that convert peptidylarginine into peptidylcitrulline, whose dysregulation has been associated with Alzheimer's disease and others neurodegenerative disorders. Against this background, the goal of this project is to characterize the citrullination during infection with HSV-1 in different *in vitro* models. We demonstrate that HSV-1 triggers PADs expression in Human Foreskin Fibroblasts (HFF), African green monkey kidney cells (VERO) and Human Neuroblastoma Cell Line (SHSY-5) cell lines both at mRNA and protein levels. Furthermore, the overall citrullination profile obtained with the citrulline-specific rhodamine phenylglyoxal (RhPG)-based probe changes consistently at different time points during infection in all tested cell lines. Finally, HSV-1 replication rate is strongly impaired in the presence of Cl-amidine and BB-Cl-amidine, two specific pan-PAD inhibitors, indicating that citrullination is required for HSV-1 replication. These findings could shed light on the role of HSV-1 in the pathogenesis of AD, providing new molecular mechanisms that could be exploited for advanced medical interventions.

Introduction

Herpes simplex virus type 1 (HSV-1) is a neurotropic virus, with a seroprevalence ranging from 60 to 90% in adults. The pathogenesis of HSV-1 infection follows a cycle of primary infection of epithelial cells usually in childhood, latency primarily in neurons, and reactivation. Recent evidence suggests that HSV-1 may be involved in the etiology of Alzheimer's disease (AD)(Itzhaki 2016). Sporadic AD is a complex multifactorial neurodegenerative disease with evidence indicating coexisting multi-pathogen and inflammatory etiologies. PADs are a family of calcium dependent enzymes that catalyze post-translational modification (PTM) citrullination, also called deimination, a process in which the guanidinium group of a peptidyl-arginine is hydrolyzed to form peptidyl-citrulline, a non-genetically coded amino acid(Witalisom, Thompson, and Hofseth 2002; Mondal and Thompson 2019). Five PAD isozymes (PADs 1-4 and 6) are expressed in humans with a unique distribution in various tissues(Vossenaar et al. 2003), whose dysregulation has been associated with AD and others neurodegenerative disorders. Moreover, a correlation has recently emerged between PADs dysregulation and viral infections. Specifically, it was found that the antiviral activity of the LL37 protein is compromised by human rhinovirus (HRV) induced citrullination(Casanova et al. 2020). Other research has shown that sera from RA patients specifically recognize artificially citrullinated Epstein-Barr virus (EBV) proteins(Trier et al. 2018; F. Pratesi et al. 2011; Federico Pratesi et al. 2006). Finally, we have recently established that human cytomegalovirus (CMV), another member of the *Herpesviridae* family, triggers PAD-mediated citrullination and this activity promotes viral fitness. Moreover, we have also shown that PAD-inhibitors inhibit CMV and HSV-1 replication *in vitro*(Griffante et al. 2021). Based on these premises, the goal of this project was to characterize the citrullination profile during HSV-1 infection in different neuronal *in vitro* models.

Results

Antiviral activity of PADs inhibitor Cl-amidine against HSV1 replication.

Human Foreskin Fibroblasts (HFFs), African green monkey kidney cells (VERO), Human Neuroblastoma Cell Line (SHSY-5Y) and Human medulloblastoma cell lines (ONS-76 and DAOY) were infected with HSV1 (MOI 0.1), and then treated with increasing concentrations of the pan-PADs inhibitor Cl-amidine. At 48 hpi, cell-free supernatants were harvested and virus amounts determined by plaque assays (Fig 1). Viral expression of immediate early (ICP0), early (HSV1 polymerase) and late (gE2) genes were analyzed by RT-PCR from infected cells (24 hpi) treated with Cl-A or BB-Cl-amidine (a different pan-PAD nhibitor) compared to infected-DMSO treated cells (Fig 2 A-D).

Although the inhibition of PADs compromises the expression of viral genes and HSV1 replication in all the cell lines tested, this reduction is much more marked in the three cell lines of neuronal origin and in particular in neuroblastoma cells (SHSY-5Y). Viral DNA was also extracted from the same samples and quantified by RT-PCR. The quantity of viral DNA is significantly lower in the samples treated with Cl-A compared to DMSO-treated cells (Fig 2 E-F).

Then we focus on the effect of pan-PAD inhibitor Cl-amidine in SHSY-5 and HFF cells. In order to analyze its antiviral effect, we performed a Western Blot of protein lysates from untreated cells or treated cells with 100µM of Cl-amidine; all of them were then infected with HSV1 (MOI=1) and harvested at three different times: 8hpi, 15hpi and 24hpi. We used two proteins to study the effect of Cl-amidine along the infection process: ICP27 as an immediate early infection control and gD as a late phase infection control. The actine protein was used as a loading control. As we can see in Fig. 2 G-H, there is a slightly downregulation of viral proteins' production compared to untreated samples. This downregulation is more prominent in samples at 15hpi. Further studies are required to clarify the antiviral effect of Cl-amidine against HSV1.

Infection with HSV1 enhances protein citrullination in different vitro models

In order to evaluate the citrullinated protein profile following HSV1 infection, we infected primary Human Foreskin Fibroblasts (HFFs), African green monkey kidney cells (VERO), Human Neuroblastoma Cell Line (SHSY-5Y) and Human medulloblastoma cell lines (ONS-76 and DAOY) with HSV1 at a multiplicity of infection (MOI) of 3 PFU/cell. We took advantage of a rhodamine phenylglyoxal-based probe (Rh-PG, Cayman Chemical, USA) (Bicker et al., 2012), which specifically binds citrulline at low pH when incubated with total cell lysates. As shown in Figure 3, we observed an increase in total protein citrullination in lysates from all cell lines infected with HSV1 at 15 and 24 hours post infection (hpi) compared to uninfected control cells (mock).

PAD2, PAD3 and PAD4 expression is induced upon HSV1 infection

Next, we sought to establish a causal link between infection and the expression of enzymes mediating enhanced citrullination. It is well established that protein citrullination is catalyzed by the protein arginine deiminase (PAD) family of enzymes. There are five PAD isozymes (PADs 1-4 and 6) that are uniquely distributed in various tissues. PADs 1-4 all possess deiminase activity, whereas PAD6 displays a number of inactivating mutations (Bicker et al., 2013; Vossenaar et al., 2003). Thus, we asked which PADs were expressed in two of our cell lines: HFFs and SHSY-5Y, and whether their expression levels would be altered upon HSV1 infection. For this purpose, we performed a Western Blot of protein lysates from uninfected (mock) or infected cells at differ time points to detect PADs,

ICP27 as infection control and Actin as a loading control. PAD2, PAD3 and PAD4 protein levels increase during HSV1 infection (MOI3) (Fig. 4). The other PAD family members, including PAD1, and PAD6, were only expressed at very low or undetectable levels and were not affected by HSV1 infection (data not shown).

PADs knock-down impairs HSV1 genes expression.

Previously we demonstrated that PAD2, PAD3 and PAD4 protein levels increase during HSV1 infection, conversely the other PAD family members, including PAD1, and PAD6, were only expressed at very low or undetectable levels and were not affected by HSV1 infection. Given the above, we next sought to determine whether targeting the enzymatic activity of PAD2, PAD3 and PAD4 would affect viral replication. To this end, SHSY-5Y cells were treated with specific inhibitors of isoforms PAD2, PAD3 and PAD4 (respectively AFM30a 20 µM, HF4 5 µM and GSK199 20 µM) or with equal volumes of DMSO solvent 1 h before infection and for the entire duration of the infection, and infected with HSV1 at a MOI of 1. Immunoblot analysis of total protein extracts showed a dramatic downregulation of viral protein expression levels in comparison with vehicle-treated infected cells (Fig. 5A) especially in cells treated with the PAD3 inhibitor. Fittingly, plaque assay on these cells confirmed a significant reduction of the viral titer in the presence of PADs inhibitors (Fig. 5B).

To further validate this results SHSY-5Y were electroporated with PAD2, PAD3 and PAD4 specific cocktail mix siRNA or siRNA control. At 24 h post electroporation, cells were infected with HSV1 (MOI=1). Protein lysates were harvested from siRNA-transfected cells at 24 hpi. Efficiency of siRNA-mediated reduction of cellular gene expression at the protein level was evaluated by WB analysis. At the same time the viral protein expression was evaluated by Immunoblot analysis. Depletion of PADs protein, especially PAD3, shows significantly decreased viral protein expression compared to the controls.

Bibliography

Wang S, Wang Y. Peptidylarginine deiminases in citrullination, gene regulation, health and pathogenesis. *Biochim Biophys Acta* 2013;**1829**:1126–35

Fuhrmann J, Clancy KW, Thompson PR. Chemical biology of protein arginine modifications in epigenetic regulation. *Chem Rev* 2015;**115**:5413–61

van Beers JJBC, Schwarte CM, Stammen-Vogelzangs J, Oosterink E, Božič B, Pruijn GJM. The rheumatoid arthritis synovial fluid citrullinome reveals novel citrullinated epitopes in apolipoprotein E, myeloid nuclear differentiation antigen, and β-actin. *Arthritis Rheum* 2013;**65**:69–80

Pruijn GJM. Citrullination and Carbamylation in the Pathophysiology of Rheumatoid Arthritis. *Front Immunol* 2015;**6:**192

Wang F, Chen F-F, Gao W-B, Wang H-Y, Zhao N-W, Xu M, Gao D-Y, Yu W, Yan X-L, Zhao J-N, Li X-J. Identification of citrullinated peptides in the synovial fluid of patients with rheumatoid arthritis using LC-MALDI-TOF/TOF. *Clin Rheumatol* 2016;**35**:2185–94

Witalison EE, Thompson PR, Hofseth LJ. Protein Arginine Deiminases and Associated Citrullination: Physiological Functions and Diseases Associated with Dysregulation. *Curr Drug Targets* 2015;**16**:700–10

Ruth F. Itzhaki Herpes and Alzheimer's Disease: Subversion in the Central Nervous System and How It Might Be Halted. *Journal of Alzheimer's Disease* 2016; 54(4):1273-1281.

Bicker KL, Subramanian V, Chumanevich AA, Hofseth LJ, Thompson PR. Seeing citrulline: development of a phenylglyoxal-based probe to visualize protein citrullination. *J Am Chem Soc* 2012;**134**:17015–8

Bicker KL, Thompson PR. The protein arginine deiminases: Structure, function, inhibition, and disease. *Biopolymers* 2013;**99**:155–63

Vossenaar ER, Zendman AJW, van Venrooij WJ, Pruijn GJM. PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease. *BioEssays News Rev Mol Cell Dev Biol* 2003;**25**:1106–18

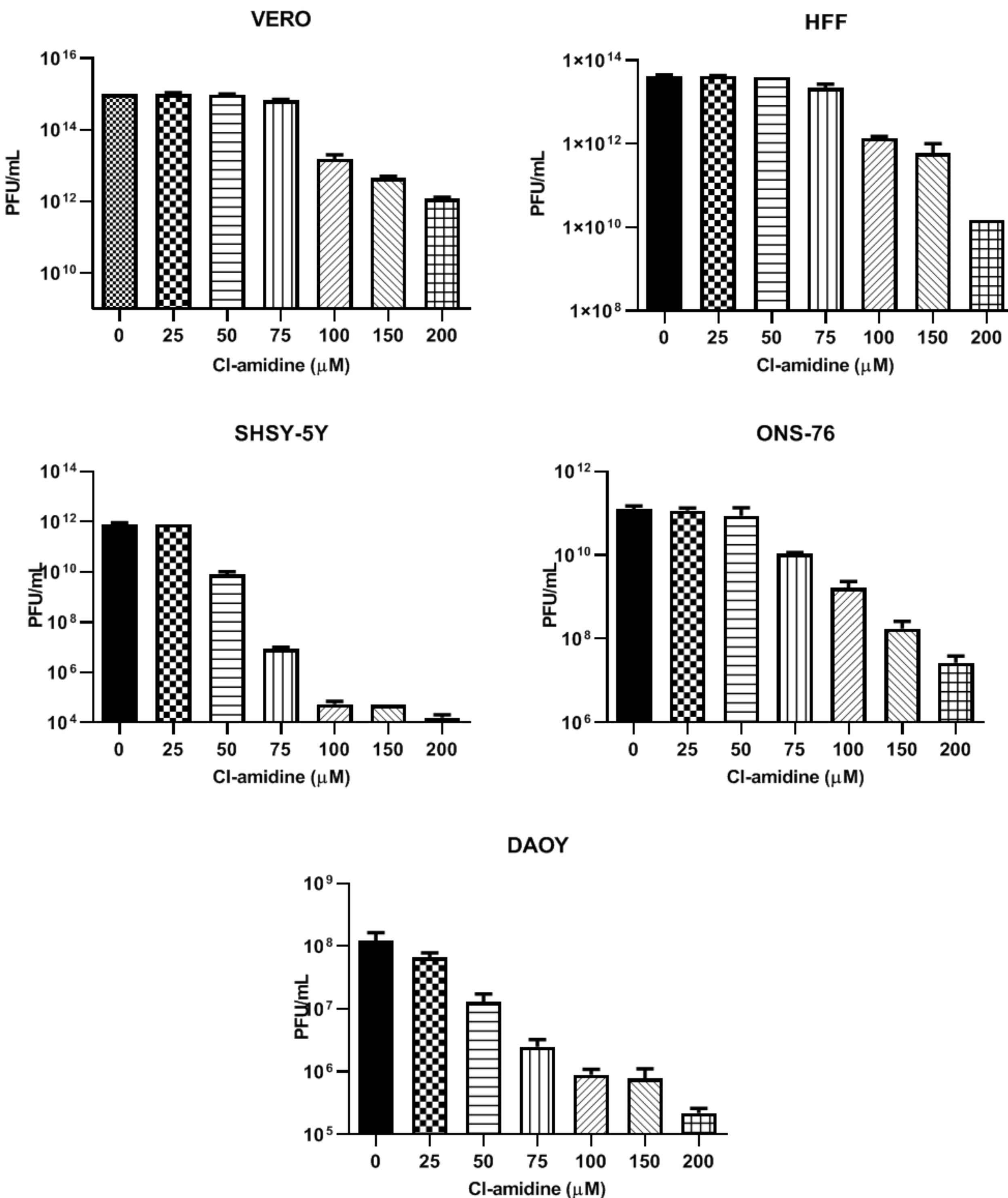


Figure 1 Five different cell lines (VERO, HFF, SHSY-5Y, ONS-76 and DAOY) were treated with increasing concentrations of inhibitors, and then infected with HSV1 (MOI=0.1). At 48hpi, viral plaques were microscopically counted and the number of plaques (Plaques Forming Units/mL) is plotted as a function of inhibitor concentration

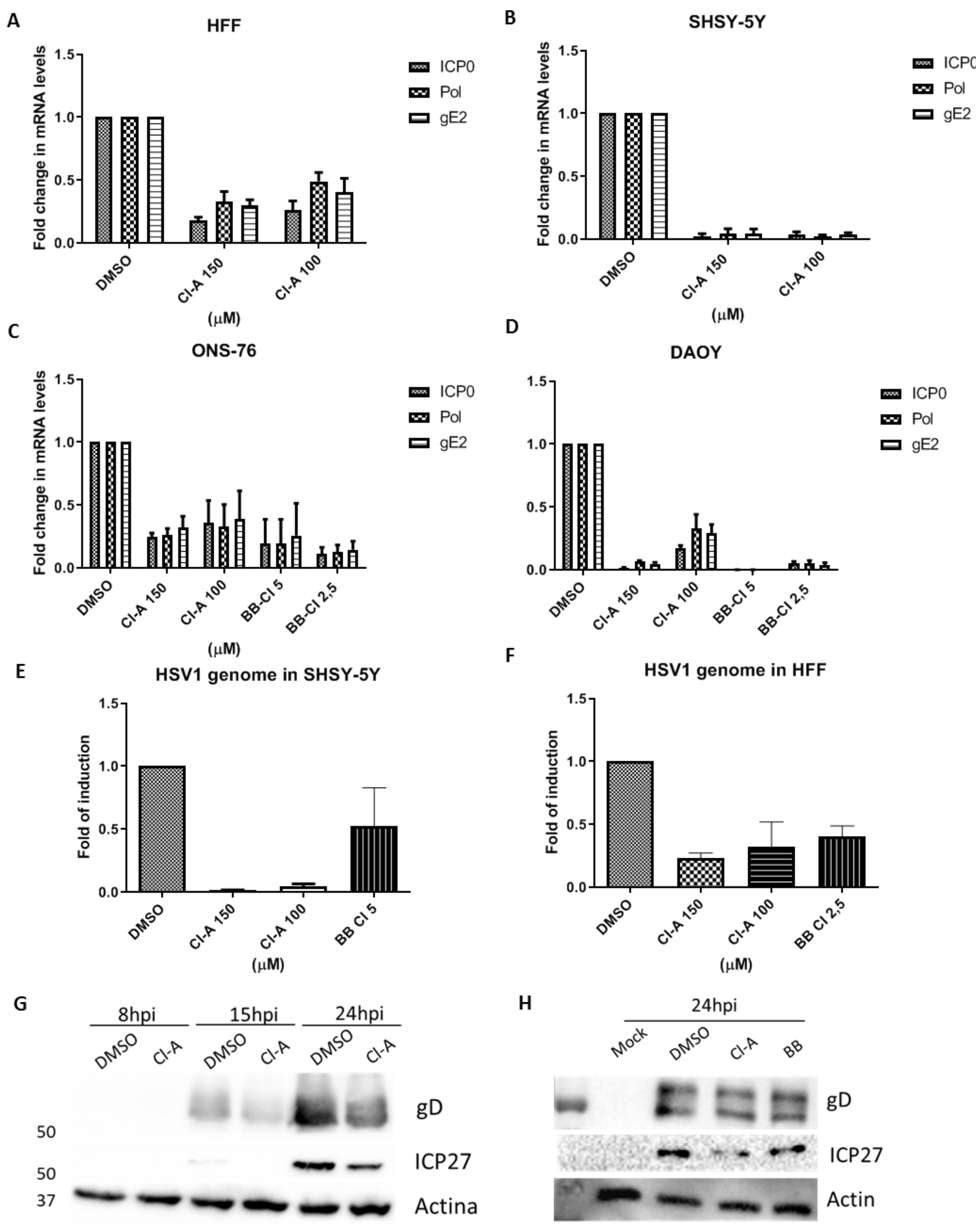


Figure 2 (A-D) mRNA expression of viral gene by qRT-PCR of HSV1-infected (24 hpi) versus uninfected (mock) cells, normalized to GAPDH expression. Viral DNA quantification by quantitative

RT-PCR in SHSY-5Y (E) or HFF (F) treated cells with Cl-Amidine or BB-cl-amidine compared to DMSO-treated cells. Western blot analysis of protein lysates from SHSY-5Y (G) or HFF (H) untreated cells (DMSO) or treated cells with 100µM or 2,5µM of Cl-Amidine or BB-cl-amidine respectively, then infected with HSV1 (MOI=1). Samples were harvested at three different time points (8hpi, 15hpi and 24hpi) and analyzed for ICP27 as immediate early infection control, gD as a late phase infection control and actin as a loading control.

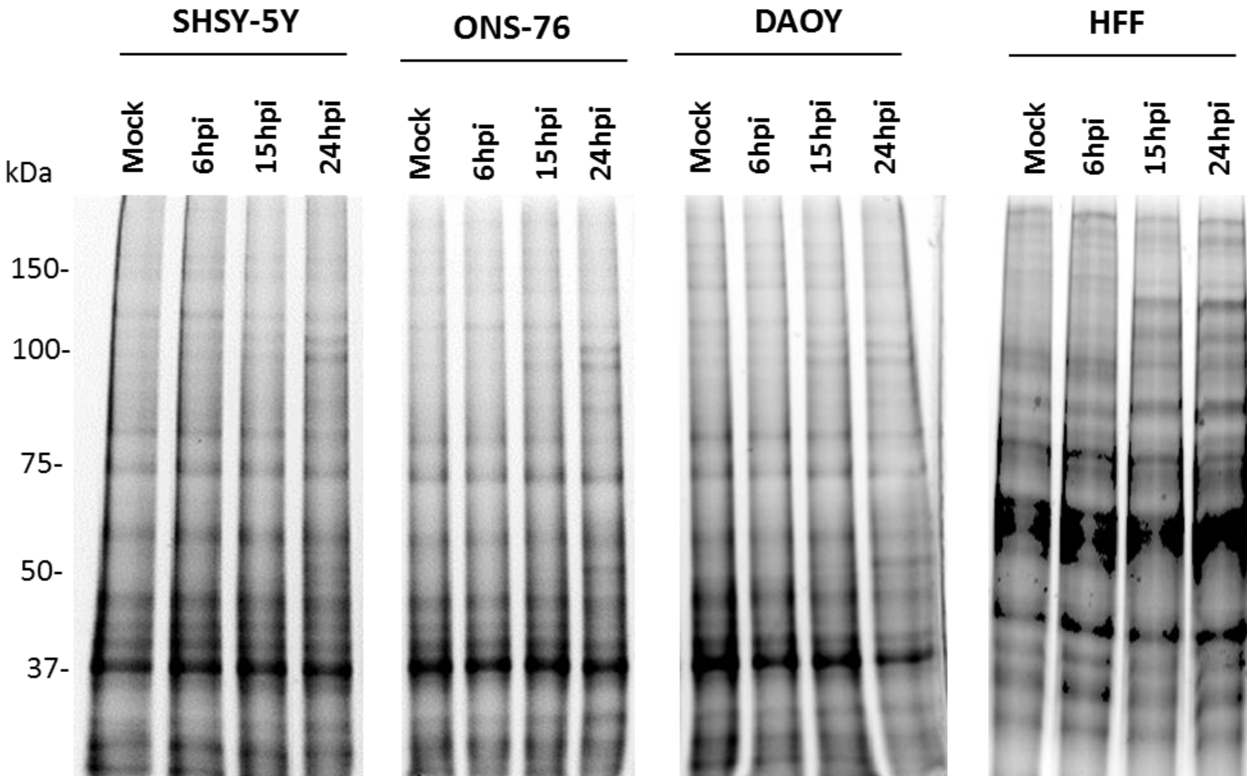


Figure 3 Protein lysates from uninfected (mock) or infected cells were exposed to a Rh-PG citrulline-specific probe and subjected to gel electrophoresis to detect total proteins citrullination.

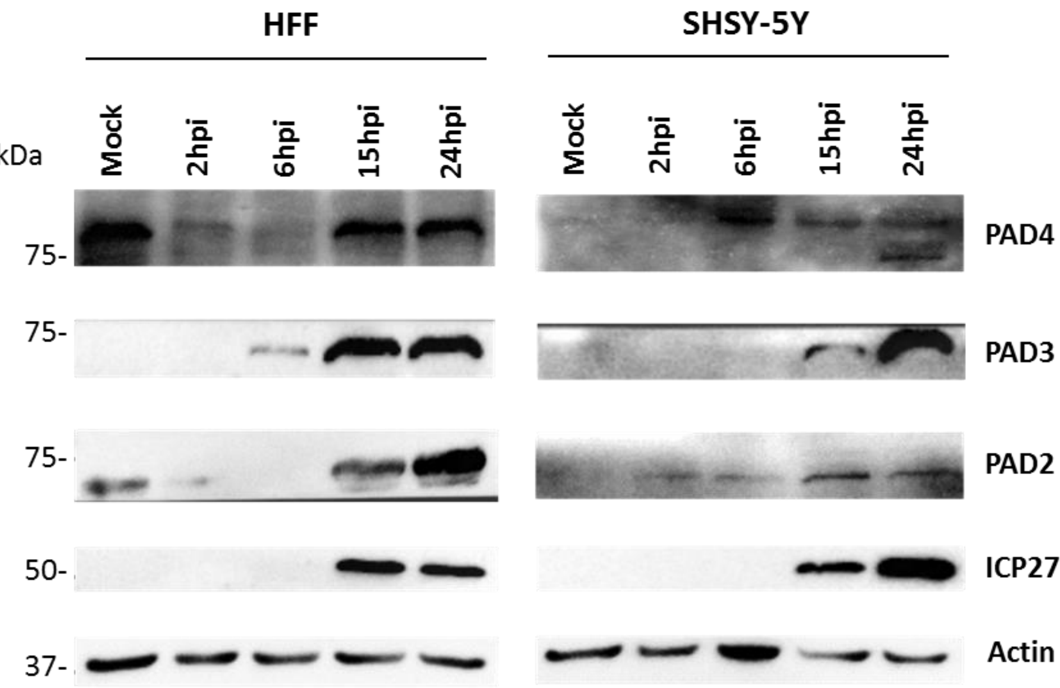


Figure 4 Western blot of protein lysates from uninfected (mock) or infected with HSV1 (MOI 3) HFFs and SHSY-5Y cells to detect PADs, ICP27 as infection control and ACTIN as a loading control.

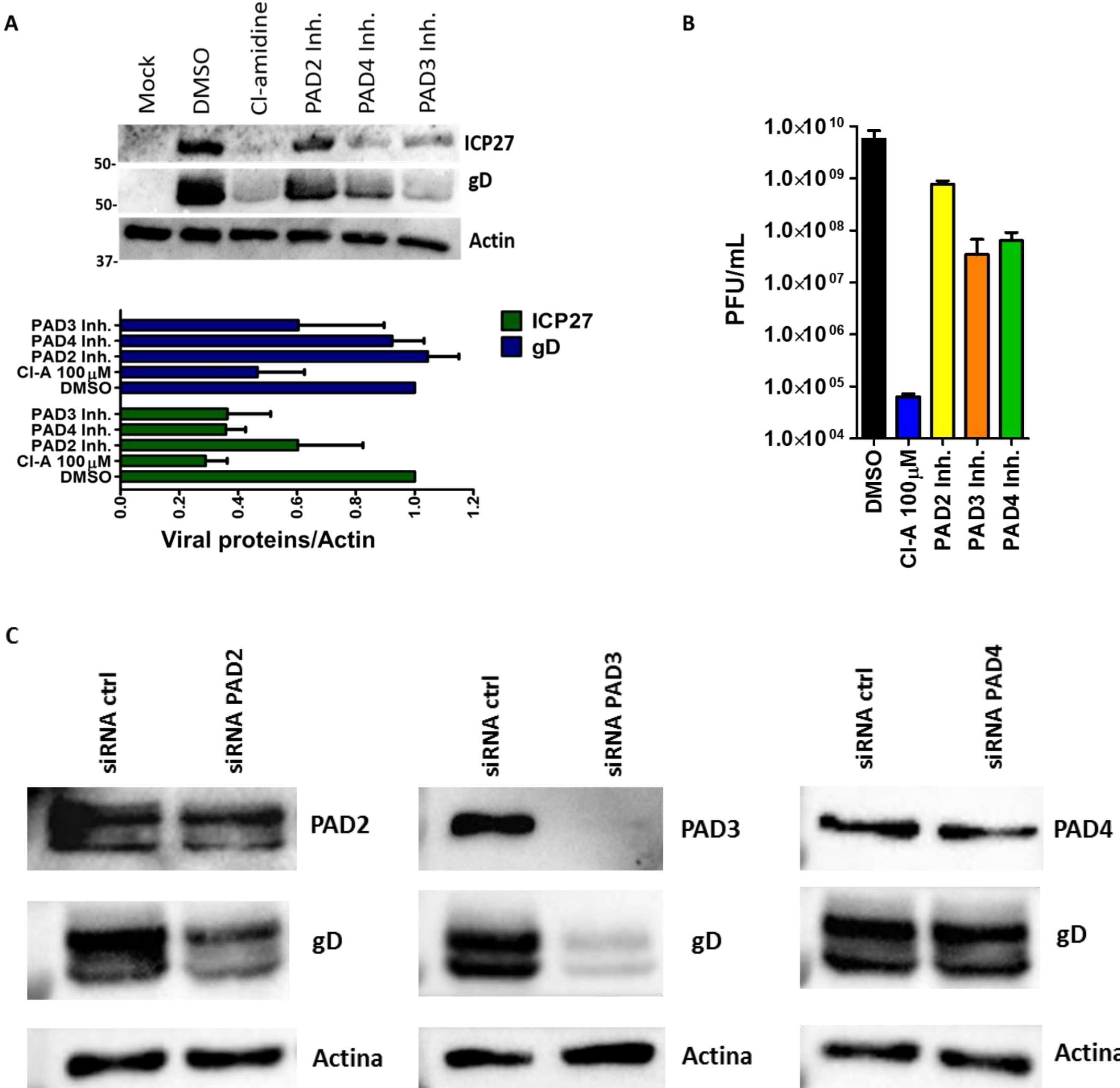


Figure 5 (A) SHSY-5Y cells were treated with specific inhibitors of isoforms PAD2, PAD3 and PAD4 (respectively AFM30a 20 µM, HF4 5 µM and GSK199 20 µM) or with equal volumes of DMSO solvent 1 h before infection and for the entire duration of the infection, and infected with HSV1 at a MOI of 1. Lysates were prepared at 24 hpi and subjected Western blot analyses. Values of densitometric analysis are expressed as mean ± SEM of two independent experiments. (B) SHSY-5Y cells were infected with HSV1 (MOI 0.1 PFU/cell) and then treated as described above. Viral productions were collected at 48 hpi and analyzed by plaque assay. Values are expressed as mean ± SEM of three independent experiments. (C) Western blot of protein lysates from HFF PADs knock-down infected whit HSV1 (24hpi, MOI 1).

Antiviral Research

Novel antiviral activity of PAD inhibitors against human beta-coronaviruses HCoV-OC43 and SARS-CoV-2

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research paper
Section/Category:	Other RNA viruses
Keywords:	SARS-CoV-2; HCoV-OC43; coronavirus; citrullination; peptidyl-arginine deiminases; host-targeting antivirals.
Corresponding Author:	Marco De Andrea University of Turin: Universita degli Studi di Torino ITALY
First Author:	Selina Pasquero
Order of Authors:	Selina Pasquero
	Francesca Gugliesi
	Gloria Griffante
	Valentina Dell'Oste
	Matteo Biolatti
	Camilla Albano
	Greta Bajetto
	Serena Delbue
	Lucia Signorini
	Maria Dolci
	Santo Landolfo
	Marco De Andrea
Manuscript Region of Origin:	Europe
Abstract:	The current SARS-CoV-2 pandemic, along with the likelihood that new coronavirus strains will appear in the nearby future, highlights the urgent need to develop new effective antiviral agents. In this scenario, emerging host-targeting antivirals (HTAs), which act on host-cell factors essential for viral replication, are a promising class of antiviral compounds. Herein, we report the discovery of a new class of HTAs endowed with a potent inhibitory activity against human beta-coronaviruses (HCoVs). Specifically, we show that infection of lung and kidney epithelial cell lines with HCoV-OC43 and SARS-CoV-2 leads to aberrant citrullination of cellular proteins, a posttranslational modification associated with various inflammatory conditions. Most importantly, we show that targeting the cellular enzymes catalyzing protein citrullination— i.e. , peptidylarginine deiminases (PADs)—significantly reduces HCoV-OC43 and SARS-CoV-2 replication in vitro . Overall, our results demonstrate the potential efficacy of PAD inhibitors in suppressing HCoV infection, which may provide the rationale for the repurposing of this class of inhibitors for the treatment of COVID-19 patients.
Suggested Reviewers:	Enzo Tramontano tramon@unica.it
	Thomas Stamminger thomas.stamminger@uniklinik-ulm.de
	Katherine Seley-Radtke kseley@umbc.edu

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

Opposed Reviewers:	

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

UNIVERSITÁ DEGLI STUDI DI TORINO – UNIVERSITY OF TURIN, MEDICAL SCHOOL
DIPARTIMENTO DI SCIENZE DELLA SANITA' PUBBLICA E PEDIATRICHE
DEPARTMENT OF PUBLIC HEALTH AND PEDIATRICS

Editor-in-Chief, *Antiviral Research*
Mike Bray, MD
Chevy Chase, MD, USA

November 29th, 2021

Dear Dr. Bray,

Please find enclosed the manuscript entitled *"Novel antiviral activity of PAD inhibitors against human beta-coronaviruses HCoV-OC43 and SARS-CoV-2"* by Pasquero *et al.*, which we wish to publish as a Research Article in Antiviral Research.

In this study, we demonstrate an unprecedented role of citrullination (*i.e.,* conversion of arginine into citrulline) in the replication of two human beta-coronaviruses (β-HCoVs), HCoV-OC43 and SARS-CoV-2, in lung and kidney epithelial cells. In particular, we provide evidence that both viruses enhance protein citrullination through upregulation of peptidyl arginine deiminases (PADs). Furthermore, we show that four well-known PAD inhibitors—*i.e.*, Cl-amidine, BB-Cl-amidine, GSK199 and AFM30a—can revert this effect, suggesting that these drugs may be repurposed to treat β-HCoV infections, such as COVID-19.

We believe that this manuscript is appropriate for publication in Antiviral Research for the following reasons: i) it identifies a previously unknown mechanism of virus-mediated citrullination exploited by β-HCoVs to foster their replication, and ii) it characterizes the *in vitro* antiviral activity of well-defined chemical compounds for the treatment of viral infection by HCoV-OC43 and SARS-CoV-2.

Overall, PAD enzymes could be potentially exploited to design new therapies against a broad spectrum of present and future coronavirus-related diseases.

All the authors have actively participated to this work, and approved the submission to Antiviral Research.

UNIVERSITÁ DEGLI STUDI DI TORINO – UNIVERSITY OF TURIN, MEDICAL SCHOOL
DIPARTIMENTO DI SCIENZE DELLA SANITA' PUBBLICA E PEDIATRICHE
DEPARTMENT OF PUBLIC HEALTH AND PEDIATRICS

We would like to take this opportunity to thank you in advance for your thoughtful consideration of our work.

Sincerely yours,

Marco De Andrea, M.D., PhD
Associate Professor of Medical Microbiology
Dept. of Public Health and Pediatrics
University of Turin, Medical School
Via Santena, 9 - 10126 Turin (ITALY)
marco.deandrea@unito.it

Highlights

- Beta-coronavirus replication induces peptidyl-arginine deiminase (PAD)-mediated citrullination *in vitro*
- The PAD inhibitors Cl-amidine and BB-Cl-amidine strongly impair HCoV-OC43 and SARS-CoV-2 replication *in vitro*
- PAD inhibitors may be repurposed for treating HCoV-associated infections

Manuscript Click here to view linked References

Novel antiviral activity of PAD inhibitors against human beta-coronaviruses HCoV-OC43 and SARS-CoV-2

Selina Pasquero[a], Francesca Gugliesi[a], Gloria Griffante[b], Valentina Dell'Oste[a], Matteo Biolatti[a], Camilla Albano[a], Greta Bajetto[a], Serena Delbue[d], Lucia Signorini[d], Maria Dolci[d], Santo Landolfo[a], Marco De Andrea[a,c]

[a] *Department of Public Health and Pediatric Sciences, University of Turin – Medical School, Turin, Italy*
[b] *Department of Translational Medicine, University of Piemonte Orientale, Novara, Italy*
[c] *CAAD Center for Translational Research on Autoimmune and Allergic Disease, University of Piemonte Orientale, Novara Medical School, Italy*
[d] *Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy*

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65

ABSTRACT

The current SARS-CoV-2 pandemic, along with the likelihood that new coronavirus strains will appear in the nearby future, highlights the urgent need to develop new effective antiviral agents. In this scenario, emerging host-targeting antivirals (HTAs), which act on host-cell factors essential for viral replication, are a promising class of antiviral compounds. Herein, we report the discovery of a new class of HTAs endowed with a potent inhibitory activity against human beta-coronaviruses (HCoVs). Specifically, we show that infection of lung and kidney epithelial cell lines with HCoV-OC43 and SARS-CoV-2 leads to aberrant citrullination of cellular proteins, a posttranslational modification associated with various inflammatory conditions. Most importantly, we show that targeting the cellular enzymes catalyzing protein citrullination—*i.e.*, peptidylarginine deiminases (PADs)—significantly reduces HCoV-OC43 and SARS-CoV-2 replication *in vitro*. Overall, our results demonstrate the potential efficacy of PAD inhibitors in suppressing HCoV infection, which may provide the rationale for the repurposing of this class of inhibitors for the treatment of COVID-19 patients.

Keywords

SARS-CoV-2, HCoV-OC43, coronavirus, citrullination, peptidyl-arginine deiminases, host-targeting antivirals.

1. Introduction

In recent years, emerging zoonotic RNA viruses have raised serious public health concerns worldwide. Among them, novel coronaviruses (CoVs) deserve special attention due to their high spillover potential and transmissibility rate, often leading to deadly epidemics across multiple countries, worsened by the lack of effective therapies (Fan et al., 2019).

The *Coronaviridae* family consists of enveloped single-stranded, positive-sense RNA viruses classified into four coronavirus genera: alpha, beta, gamma, and delta. To date, seven human coronaviruses (HCoVs), belonging to the alpha and beta genera, have been identified (Su et al., 2016). HCoV-229E and HCoV-OC43 were first described in 1966 and 1967, respectively, followed by HCoV-NL63 in 2004 and HCoV-HKU1 in 2005. HCoVs generally establish infections in the upper respiratory district—responsible for about 10-30% of common cold cases—, but in vulnerable patients they can also cause bronchiolitis and pneumonia (Leao et al., 2020; Paules et al., 2020).

Even though HCoVs have long been recognized as human pathogens, effective treatments against these viruses have only started to be developed after the severe acute respiratory syndrome CoV (SARS-CoV) outbreak in 2002 (Ksiazek et al., 2003; Weiss and Navas-Martin, 2005). Since then, recurrent spillover events from wildlife have led to the appearance of two other highly pathogenic beta-CoV strains associated with severe respiratory diseases in humans: the Middle East respiratory syndrome coronavirus (MERS-CoV) in 2011, which causes MERS (De Wit et al., 2016; Zaki et al., 2012), and the severe acute respiratory syndrome CoV-2 (SARS-CoV-2) in 2019, the etiological agent of the ongoing pandemic of coronavirus disease 2019 (COVID-19) (Lu et al., 2020; Wu et al., 2020).

In this scenario, the widespread vaccine hesitancy, the growing number of breakthroughs among the vaccinated population, the emergence of increasingly infectious SARS-CoV-2 variants, and the likelihood that new CoV strains will continue to appear in the future have all led to the urgent need to develop new antiviral agents able to tackle ongoing SARS-CoV-2 outbreaks. Consistent with this emergency status, HCoV-OC43 has often been used as a surrogate of—or together with—SARS-

CoV-2 to test potential inhibitors of HCoV replication in both cell-based assays and *in silico* analysis (Milani et al., 2021)

Most of the approved antiviral drugs are the so-called direct-acting antiviral agents (DAAs), compounds designed against viral proteins deemed essential for infection. For example, remdesivir, whose efficacy against SARS-CoV-2 is highly controversial (Hsu, 2020), and molnupiravir, a new oral antiviral highly effective in preventing severe disease based on the results of a recent Phase 2a trial (Fischer et al., 2021), are nucleoside analogue prodrugs acting as competitive substrates for virally-encoded RNA-dependent RNA polymerase (RdRp) (Beigel et al., 2020; Warren et al., 2016). Another emerging class of antiviral agents named host-targeting antivirals (HTAs) consists of drugs acting on host-cell factors involved in viral replication. To date, most studies have focused on the analysis of viral proteins and the identification of potential DAAs. However, viruses encode a limited number of proteins, and those suitable as drug targets are only a subset of them. Therefore, targeted disruption of the mechanisms devised by HCoVs to manipulate the host cellular environment during infection, such as those leading to immune evasion and host gene expression alterations (Hartenian et al., 2020), holds great promise for the treatment of COVID-19 patients.

Peptidyl-arginine deiminases (PADs) are a family of calcium-dependent enzymes that catalyze a posttranslational modification (PTM) named citrullination, also known as deimination, a process during which the guanidinium group of a peptidyl-arginine is hydrolyzed to form peptidyl-citrulline, an unnatural amino acid (Mondal and Thompson, 2019; Witalisom et al., 2002). Five PAD isozymes (PAD 1-4 and 6) are expressed in humans, with a unique distribution in various tissues (Vossenaar et al., 2003). PAD dysregulation leads to aberrant citrullination, which is a typical biomarker of various inflammatory conditions, suggesting that it may play a pathogenic role in inflammation-related diseases (Acharya et al., 2012; Knight et al., 2015; Sokolove et al., 2013; van Venrooij et al., 2011; Yang et al., 2016; Yuzhalin, 2019).

In this scenario, a correlation between PAD dysregulation and viral infections has recently emerged. In particular, the antiviral activity of the LL37 protein appears to be compromised upon

human rhinovirus (HRV)-induced citrullination (Casanova et al., 2020), and sera from RA patients can specifically recognize artificially citrullinated Epstein-Barr virus (EBV) proteins (Pratesi et al., 2011, 2006; Trier et al., 2018). Consistently, we have recently shown that human cytomegalovirus (HCMV) induces PAD-mediated citrullination of several cellular proteins endowed of antiviral activity, including the two IFN-stimulated genes (ISGs) *IFIT1* and *Mx1*, and that the inhibition of this process by the PAD inhibitor Cl-amidine blocks viral replication (Griffante et al., 2021). Finally, another recent study has shown that SARS-CoV-2 infection can modulate PADI gene expression, particularly in lung tissues, leading to the intriguing possibility that PAD enzymes may play a critical role in COVID-19 disease (Arisan et al., 2020).

Based on this evidence, the aim of this work was to ascertain whether PAD inhibitors might constitute a new class of HTAs against HCoVs. For this purpose, we performed cell based-assays to measure the antiviral activity of various well-characterized PAD inhibitors against two members of the beta-CoV genus: HCoV-OC43, the first one to have been discovered, and SARS-CoV-2, the last one to have emerged so far.

Overall, our results show that both HCoV-OC43 and SARS-CoV-2 infections are significantly associated with PAD-mediated citrullination *in vitro*. Importantly, pharmacological inhibition of PAD enzymes inhibits SARS-CoV-2 replication, suggesting that PAD inhibitors may be repurposed to treat COVID-19.

2. Materials and methods

2.1 Cell lines and viruses

Human lung fibroblast MRC-5 cells (ATCC® CCL-171) and African green monkey kidney Vero-E6 cells (ATCC®-1586) were propagated in Dulbecco's Modified Eagle Medium (DMEM; Sigma) supplemented with 1% (v/v) penicillin/streptomycin solution (Euroclone) and heat-inactivated 10% (v/v) fetal bovine serum (FBS) (Sigma). The human coronavirus strain OC43

(HCoV-OC43) (ATCC® VR-1558) was kindly provided by Dr. David Lembo (Department of Clinical and Biological Sciences, University of Turin, Turin, Italy). HCoV-OC43 was propagated on MRC-5 cells at 33°C in a humidified 5% CO_2 incubator and titrated by standard plaque method on MRC-5 cells, as described elsewhere (Marcello et al., 2020). SARS-CoV-2 was isolated from a nasal-pharyngeal swab positive for SARS-CoV-2. The complete nucleotide sequence of the SARS-CoV-2 isolated strain was deposited at GenBank, at NCBI and at GISAID (accession number: GenBank: MT748758.1; GISAID EPI_ISL 584051).

2.2 Reagents and treatments

The PAD inhibitors Cl-amidine (Cl-A), BB-Cl-amidine (BB-Cl), GSK199, and AFM30a—also known as CAY10723—were from Cayman Chemical (Ann Arbor). The compounds were incubated with cells for 1 h prior to infection and kept throughout the whole experiment.

2.3 Cell viability assay

MRC-5 or Vero-E6 cells were seeded at a density of $3*10^4$/well in a 96-well plate. After 24 h, cells were treated with different dilutions of the indicated compound or mock-treated using the vehicle alone (DMSO). Seventy-two h after treatment, cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) method previously described (Griffante et al., 2021).

2.4 In vitro antiviral assay

MRC-5 and Vero-E6 cells were cultured in a 24-well plate for 1 day and then incubated with the aforementioned PAD inhibitors at the indicated concentrations for 1 h. Subsequently, cells were infected with HCoV-OC43 at a multiplicity of infection (MOI) of 0.1. Following virus adsorption (2

h at 33°C), the viral inoculum was removed, and the cell cultures were maintained in medium containing the indicated treatment for 72 h. DMSO was used as negative control. The extent of HCoV-OC43 replication in MRC-5 cells was assessed by titrating the infectivity of supernatants using plaque assay and comparative real-time PCR. For Vero-E6 cells, the extent of HCoV-OC43 replication was assessed by titrating the infectivity of supernatants using comparative real-time PCR.

SARS-CoV-2 *in vitro* infection of Vero-E6 cells and the anti-viral inhibition assay was conducted as described previously (Parisi OI et al., 2021).

2.5 Plaque assay

MRC-5 cells were inoculated with 10-fold serial dilutions of the HCoV-OC43. Twenty-four h later, cells were fixed with cold acetone-methanol (50:50) and subjected to indirect immunostaining with an anti-NP-HCoV-OC43 monoclonal antibody (Millipore MAB9012). To determine the virus titer, the number of immunostained foci was counted on each well using the following formula: virus titer (PFU/ml) = number of plaques * 0.1 ml/dilution fold. SARS-CoV-2 plaque assay were performed on VERO-E6 cells as described previously (Parisi OI et al., 2021).

2.6 Comparative real-time PCR (viral load)

All molecular analyses were performed according to Milewska *et al*. (Milewska et al., 2016). Briefly, viral nucleic acids were isolated from 200 µl of sample using the TRI Reagent solution (Sigma-Aldrich), according to the manufacturer's instructions. Extracted viral RNA (4 µl per sample) was retrotranscribed and amplified in a 20 µl reaction mixture containing Sensi Fast Probe No Rox One step kit (Bioline) using a CFX Touch Real Time PCR Detection System (BioRad). The primers and probe for N gene amplification (Eurofins) are reported below:

HCoV-OC43 Fw: AGCAACCAGGCTGATGTCAATACC;

HCoV-OC43 Rv: AGCAGACCTTCCTGAGCCTTCAAT;

Probe (HCoV-OC43P_rt): TGACATTGTCGATCGGGACCCAAGTA (5' FAM and 3'TAMRA labeled).

The reaction conditions were as follows: 10 min at 45°C and 20 min at 95°C, followed by 40 cycles of 5 sec at 95°C and 1 min at 60°C.

Quantification of SARS-CoV-2 copy numbers in cell supernatants was evaluated *via* specific qRT-PCR of the *N1* gene, according to the protocols "Coronavirus disease (COVID-19) technical guidance: Laboratory testing for 2019-nCoV in humans" (WHO, 2020) and "CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel" (CDC, 2020), available at: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/laboratory-guidance and https://www.fda.gov/media/134922/download [last access 25 October 2021]), respectively.

2.7 Cell-associated RNA isolation and quantitative nucleic acid analysis

Total RNA was extracted using the TRI Reagent solution (Sigma-Aldrich), and 1 µg of it retrotranscribed using the RevertAid H-Minus FirstStrand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Comparison of mRNA expression between treated and untreated samples was performed by SYBR green-based RT-qPCR by Mx3000P apparatus (Santa Clara), using the primers reported previously. As cellular reference, we amplified the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GADPH) with the following primers: GAPDH Fw: AGTGGGTGTCGCTGTTGAAGT; GADPH Rv: AACGTGTCAGTGGTGGACCTG. The reaction conditions were as follows: 2 min at 95°C, followed by 40 cycles of 5 sec at 95°C and 1 min at 60°C.

2.8 Western blot analysis

MRC-5 or Vero-E6 cells were treated with the indicated compound or equal volumes of DMSO solvent 1 h before infection and throughout the entire duration of the infection. Cells were infected with either HCoV-OC43 or SARS-CoV-2 at an MOI of 1. Cells were harvested at the indicated time, and lysates were prepared and subjected to Western blot analysis. The primary antibodies were as follows: anti-HCoV-OC43 (Millipore MAB9012); anti-PAD1 (ABCAM); anti-PAD2 (Cosmo Bio); anti-PAD3 (ABCAM); anti-PAD4 (ABCAM); anti-PAD6 (ABCAM); anti-ACTIN (Sigma Aldrich), anti-SARS-CoV-2 (GeneTex).

2.9 Detection of citrullination with rhodamine-phenylglyoxal (Rh-PG)

Whole-cell protein extracts were prepared and subjected to immunoblotting as previously described. Equal amounts of protein were diluted with trichloroacetic acid and incubated with rhodamine phenylglyoxal (Rh-PG, Cayman) probe as described in (Griffante et al., 2021).

2.10 Statistical analysis

All data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA). All results are presented as means ± SEM. The half-maximal inhibitory concentrations (IC_{50}) and half-maximal cytotoxic concentration (CC_{50}) values were calculated by Quest Graph™ IC50 Calculator (AAT Bioquest, Inc, https://www.aatbio.com/tools/ic50-calculator). The selectivity index (SI) values were calculated as the ratio of CC_{50} and IC_{50} (SI= CC_{50}/ IC_{50}). The P-value was calculated by comparing between % inhibition of infected-treated samples and % inhibition of infected-untreated samples. One-tailed Student's t-test was used to compare groups. Significance was considered statistically significant at a P-value < 0.05 (*), < 0.01 (**), < 0.001 (***) and < 0.0001 (****).

3. Results

3.1. PAD inhibition blocks HCoV-OC43 replication in MRC-5 cells

We previously demonstrated that HCMV triggers PAD-mediated citrullination to promote its replication (Griffante et al., 2021). To evaluate whether the protein citrullination profile would also be altered during HCoV infection, we first performed an electrophoresis analysis of protein lysates obtained from MRC-5 lung fibroblasts infected with HCoV-OC43 (MOI 1) incubated with the citrulline-specific probe Rh-PG. At 48 and 72 h post infection (hpi), HCoV-OC43-infected MRC5 cells, but not mock-infected cells, showed a robust increase in total protein citrullination (Fig. 1A). Of note, the expression of viral nucleoprotein (OC43 NP) was only detected in HCoV-OC43-infected MRC5 cells, confirming the successful infection of these cells (Fig. 1A).

Next, to test whether PAD enzymatic activity plays a functional role in HCoV-OC43 replication, we treated infected cells with the two pan-PAD inhibitors Cl-A and BB-Cl and assessed viral RNA synthesis by RT-PCR. Incubation of HCoV-OC43-infected MRC5 cells with increasing amounts of the inhibitors led to a dose-dependent reduction of viral genome copies, a drop that became statistically significant at 50 μM for Cl-A and 0.5 μM for BB-Cl (Fig. 1B and 1C). The calculated IC_{50} for Cl-A and BB-Cl were 34.76 μM and 0.54 μM, respectively. To rule out compound cytotoxicity, HCoV-OC43-infected MRC-5 cells were subjected to MTT assay. The results shown in Fig. S1A, B demonstrate that none of the PAD inhibitors significantly reduced cell viability at the two aforementioned IC_{50}. Table 1 shows the CC_{50} for Cl-A (949.14 μM) and BB-Cl (10.12 μM). Based on the calculated IC_{50} and CC_{50}, the SIs of Cl-A and BB-Cl in HCoV-OC43-infected MRC-5 were quite similar and both greater than 10 (27.3 and 18.6, respectively). Consistent with the results obtained measuring viral RNA, Cl-A and BB-Cl drastically reduced both intra- and extra-cellular viral genome copy numbers (Fig. 1D), confirming the key role of citrullination in HCoV-OC43 replication and viral cycle.

To determine whether PAD inhibition would prevent viral replication and/or the production of infectious viral particles, we assessed HCoV-OC43 NP expression by Western blotting and the virus yield by plaque assay using the cell extracts and supernatants of HCoV-OC43-infected MRC-5 cells treated with 100 μM Cl-A and 2.5 μM BB-Cl, as previously described. Interestingly, both Cl-A

and BB-Cl treatments significantly reduced HCoV-OC43 NP expression in the very same total protein extracts in which a partial suppression of the citrullination profile was also observed by Rh-PG (Fig. 1E). Moreover, the two drugs significantly reduced PFUs per mL of supernatant by more than 2 and 1 logs, respectively (Fig. 1F).

3.2. PAD4 plays a central role in HCoV-OC43 replication

To gain more insight into the mechanism of HCoV-OC43-induced cellular citrullination, we asked which of the five known PAD isoforms (PAD1-4 and PAD6) would be preferentially modulated following HCoV-OC43 infection. To answer this question, we performed immunoblot analysis on whole protein lysates obtained from mock and HCoV-OC43-infected MRC5 cells collected at different time-points after infection (Fig. 2A). PAD2 and PAD4 were the only two PAD isoforms expressed in these cells, with PAD4 being the only one induced upon infection, as judged by densitometry. By contrast, PAD1, 3, and 6 were neither detectable in mock cells nor induced upon infection (Fig. 2A).

Given the above, we next sought to determine whether targeting the enzymatic activity of PAD4 would affect viral replication. To this end, OC43-infected MRC-5 cells were treated with increasing concentrations of the PAD4-specific inhibitor GSK199 or the PAD2-specific inhibitor AFM30a, as negative control, and assessed for their antiviral activity. As expected, AFM30a only partially suppressed the HCoV-OC43 replication rate—never exceeding 40% inhibition within the range of concentrations tested (Fig. 2B). In contrast, GSK199 treatment hampered viral genome production in a dose-dependent manner (IC_{50}= 0.6 μM), achieving a complete block of viral replication at 20 μM (Fig. 2C). Furthermore, MRC-5 cells treated with 20 μM GSK199 were viable, ruling out any unspecific effect due to compound toxicity (Fig. S2). Of note, we could only observe significant cytotoxicity of both compounds at concentrations above 100 μM (data not shown). This conferred them an SI > 10, which was particularly robust in the case of GSK199 (224.94) (Table 1).

To confirm these results, we measured the number of viral genome copies in cell lysates and supernatants from MRC-5 cells treated with 20 μM AFM30a or GSK199 and infected with HCoV-OC43. As expected, inhibition of PAD4 by GSK199 drastically reduced the relative viral genome copy number in both compartments compared to vehicle-treated cells, while PAD2 inhibition by AFM30a led to a much less pronounced reduction of viral genome (Fig. 2D). Consistently, immunoblot analysis of total protein extracts from HCoV-OC43-infected MRC-5 cells treated with GSK199 showed a dramatic downregulation of OC43 NP protein expression levels in comparison with vehicle-treated infected cells (Fig. 2E). In contrast, treatment with the PAD2 inhibitor AFM30a only led to a slight decrease in NP protein levels. Fittingly, plaque assay on these cells confirmed a significant reduction of the viral titer in the presence of GSK199 (~2-log reduction), while the inhibitory activity of AFM30a at the same concentration was barely detectable (Fig. 2F).

Taken together, these results suggest that PAD4 plays a major role in HCoV-OC43 replication, and that PAD4 inhibitors are promising anti-HCoV compounds.

3.3. PAD inhibitors affect HCoV-OC43 and SARS-CoV-2 replication in Vero-E6 cells

Since Vero-E6 cells represent a widely used cellular system to study beta-CoV replication in the presence of candidate antiviral compounds, we sought to extend our results also to this model. Initially, we performed a quantitative analysis of HCoV-OC43 viral RNA production at 72 hpi using different concentrations of Cl-A (50-300 μM) and BB-Cl (5-20 μM). As depicted in Fig. 3, we observed a marked reduction of viral genome replication in Vero-E6 cells treated with either 150-300 μM Cl-A (panel A) or 20 μM BB-Cl (panel B) compared to their vehicle-treated counterparts. These pronounced effects were not a consequence of an intrinsic cytotoxicity of the PAD inhibitors as none of the screened compounds significantly reduced cell viability at the same concentrations as those used in the antiviral assays (Fig. S3A, B and Table 1).

Next, to evaluate whether HCoV-OC43 infection would also trigger protein citrullination in Vero-E6 cells, we performed electrophoresis analysis of protein lysates obtained from cells infected

with HCoV-OC43 using the Rh-PG probe. As shown in Fig. 3C, protein citrullination of HCoV-OC43-infected Vero-E6 cells was significantly induced at 48 hpi, whereas it remained almost unchanged in infected cells treated with 300 μM of the Cl-A inhibitor.

To extend our findings to other beta-CoVs, we examined the impact of PAD inhibitors treatment on SARS-CoV-2 viral genome replication. As shown in Figs. 3D and 3E, both Cl-A and BB-Cl treatments suppressed SARS-CoV-2 viral genome replication in a dose-dependent manner, albeit to a lower extent than that observed for HCoV-OC43.

To characterize the protein citrullination profile during SARS-CoV-2 infection, protein lysates obtained from Vero-E6 cells infected with SARS-CoV-2, treated with or without Cl-A, were analyzed by Rh-PG (Fig. 3F). The citrullination profile of these SARS-CoV-2-infected cells was consistent with that observed upon HCoV-OC43 infection, both displaying a signal lower than 50 kDa, which specifically appeared after the infection. However, unlike what we observed in HCoV-OC43-infected cells, the citrullination signal, albeit strongly reduced, it never completely disappeared following Cl-A treatment. In line with this observation, Cl-A treatment led to ~50% reduction of SARS-CoV-2 NP protein expression (Fig. 3F). To corroborate these results, we carried out plaque reduction assays in SARS-CoV-2-infected Vero-E6 cells treated with 300 μM Cl-A as described above. Consistent with our previous results, the inhibition of PAD catalytic activity resulted in a reduction of 1 log in SARS-CoV-2 yield (Fig. 3G).

Altogether, our results demonstrate that beta-CoV infection is associated with protein citrullination. Moreover, treatment of HCoV-OC43 and SARS-CoV-2-infected cells with PAD pan-inhibitors inhibits virus replication restoring the physiological citrullination profile.

4. Discussion

We have recently shown that HCMV infection triggers PAD-mediated citrullination of several host proteins in primary human fibroblasts, and that this activity enhances viral fitness *per se* (Griffante et al., 2021). Here, we extend those findings to two RNA viruses, HCoV-OC43 and SARS-

CoV-2, which we demonstrate to be both capable of promoting PAD-mediated citrullination *in vitro*. In particular, we show that HCoV-OC43 infection of MRC-5 lung fibroblasts upregulates PAD4-mediated citrullination of proteins, and that this process is required for optimal viral replication. A similar induction in citrullination levels was also found in CoV-infected Vero-E6 cells, a non-human model system widely used to study HCoVs, especially SARS-CoV-2 (Dittmar et al., 2021; Ghosh et al., 2021; Milani et al., 2021; Wing et al., 2021), suggesting that HCoVs can modulate citrullination across species.

Citrullination is a posttranslational modification mediated by PAD family members, whose distribution and expression patterns are modulated by inflammatory signals in a tissue-specific manner (Acharya et al., 2012; Knight et al., 2015; Vossenaar et al., 2003; Willis et al., 2011; Yang et al., 2016). Fittingly, we and others have recently reported a positive association between viral infection and PAD-mediated upregulation of citrullination in different cellular models (Arisan et al., 2020; Casanova et al., 2020; Griffante et al., 2021), raising the important question as to whether pharmacological inhibition of PAD activity can be used to reduce viral replication in infected patients. In support of this possibility, we found that treatment of HCMV-infected HFFs with the pan PAD inhibitor Cl-A downregulated PAD2 and 4-mediated citrullination of cellular proteins, thereby blocking viral replication *in vitro* (Griffante et al., 2021). In the present study, we provide additional evidence supporting the use of PAD inhibitors to curb viral growth. Specifically, we show that treatment of HCoV-OC43-infected MRC-5 cells with either of the two pan-PAD inhibitors Cl-A and BB-Cl (Biron et al., 2016; Knight et al., 2015; Ledet et al., 2018; Willis et al., 2011) or the specific PAD4 inhibitor GSK199, but not the PAD2 inhibitor AFM30a, can efficiently inhibit viral replication. This finding is consistent with the fact that MRC-5 cells express basal levels of both PAD2 and PAD4, but only PAD4 is significantly upregulated upon viral infection. Furthermore, the observation that Cl-A or BB-Cl treatment markedly reduces the presence of viral genome copies in both cellular extracts and supernatants indicates that these compounds not only block viral replication but also reduce the production of infectious particles. Of note, the considerable reduction in viral

replication and titer by the GSK199 compound was achieved at concentrations that do not affect cell viability. Indeed, the high SI of GSK199 (224.94, Table 1) along with its potent antiviral activity makes this PAD4 inhibitor an attractive target for further therapeutic development, a possibility further supported by results showing that targeting PAD4 led to a significant improvement of the clinical and histopathological end-points in a preclinical model of murine arthritis (Willis et al., 2017). Another important aspect that supports the repurposing of PAD inhibitors for antiviral therapy is that their efficacy in treating various inflammatory conditions, such as arthritis, colitis, and sepsis, has already been confirmed in preclinical and *in vitro* studies, all showing a good safety profile of such compounds (Chumanevich et al., 2011; Willis et al., 2011; Zhao et al., 2016).

Our results, showing a strong induction of citrullination levels in SARS-CoV-2-infected Vero-E6 cells also highlight the potential use of PAD inhibitors to treat COVID-19 patients. It is in fact conceivable to envisage an association between SARS-CoV-2 infection and aberrant citrullination as a way to induce an inflammatory state in different tissues (Delorey et al., 2021). This would be supported by a recent study by Arisan and co-workers showing that SARS-CoV-2 infection can modulate PADI gene expression in lung tissues (Arisan et al., 2020). In good agreement, here we show that the inhibitory activity of Cl-A and BB-Cl is not only restricted to HCoV-OC43-infected fibroblasts, but it can also be extended to SARS-CoV-2-infected Vero-E6 cells, highly permissive cells commonly used to propagate and study beta-CoV strains (Ogando et al., 2020). In these cells, both compounds efficiently suppress SARS-CoV-2 genome replication in a dose-dependent manner, albeit to higher concentrations than those used to inhibit HCoV-OC43 replication. Further experiments are ongoing to test whether this difference is virus-dependent or cell line-dependent. However, also in this case the concentrations of Cl-A (150-300 μM) or BB-Cl (20 μM) capable of reducing of about 1 log the SARS-CoV-2 yield in Vero-E6 cells were not associated with significant cytotoxic effects. Similar to what seen for HCoV-OC43-infected MRC-5 cells, inhibition of PAD activity counteracted SARS-CoV-2-induced citrullination.

In the current pandemic of COVID-19, it is more important than ever that the most promising anti-SARS-CoV-2 drug candidates enter clinical development. Based on some similarities between the clinical outcome observed in autoimmune/autoinflammatory disease and COVID-19, including lung involvement and aberrant cytokine release, pan- and specific-PAD inhibitors—i.e., GSK199—repurposing can be foreseen as a valuable strategy, as it enables accelerating the use of compounds with already known safety profiles. Moreover, treatments based on molecules with beneficial multi-target activities—a concept known as polypharmacology (Ravikumar and Aittokallio, 2018)—, which may help to counteract multiple complications as those observed in COVID-19 patients, may show an increased antiviral spectrum.

In conclusion, our findings unveil an unprecedented role of citrullination in the replication of the two human coronaviruses HCoV-OC43 and SARS-CoV-2 in lung and kidney epithelial cells. We also provide evidence that increased PAD activity is required for β-HCoV replication, highlighting the potential use of PAD inhibitors as novel HTAs against β-HCoV infections. Experiments are ongoing to test the anti-β-HCoV efficacy and safety of other PAD inhibitors. Moreover, based on the current availability of different animal models to be exploited for SARS-CoV-2 replication (Lee and Lowen, 2021), including K18-hACE2 transgenic mice challenged with SARS-CoV-2 (Conforti et al., 2021), investigations are being performed to test the feasibility of our drug repurposing strategy *in vivo*.

Acknowledgments

We thank Marcello Arsura for critically reviewing the manuscript. This research was supported by the University of Turin (PoC - TOINPROVE/2020 to M.D.A., RILO2020 and RILO2021 to M.D.A., F.G., V.D.O. and M.B.), by the Ministry of Education, University and Research – MIUR (PRIN Project 2017ALPCM to V.D.O.) and by the AGING Project—Department of Excellence—Department of Translational Medicine, University of Piemonte Orientale (G.G.).

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Bibliography

Acharya, N.K., Nagele, E.P., Han, M., Coretti, N.J., DeMarshall, C., Kosciuk, M.C., Boulos, P.A., Nagele, R.G., 2012. Neuronal PAD4 expression and protein citrullination: Possible role in production of autoantibodies associated with neurodegenerative disease. Journal of Autoimmunity 38, 369–380. https://doi.org/10.1016/j.jaut.2012.03.004

Arisan, E.D., Uysal-Onganer, P., Lange, S., 2020. Putative roles for peptidylarginine deiminases in COVID-19. International Journal of Molecular Sciences 21, 1–29. https://doi.org/10.3390/ijms21134662

Beigel, J.H., Tomashek, K.M., Dodd, L.E., Mehta, A.K., Zingman, B.S., Kalil, A.C., Hohmann, E., Chu, H.Y., Luetkemeyer, A., Kline, S., Lopez de Castilla, D., Finberg, R.W., Dierberg, K., Tapson, V., Hsieh, L., Patterson, T.F., Paredes, R., Sweeney, D.A., Short, W.R., Touloumi, G., Lye, D.C., Ohmagari, N., Oh, M., Ruiz-Palacios, G.M., Benfield, T., Fätkenheuer, G., Kortepeter, M.G., Atmar, R.L., Creech, C.B., Lundgren, J., Babiker, A.G., Pett, S., Neaton, J.D., Burgess, T.H., Bonnett, T., Green, M., Makowski, M., Osinusi, A., Nayak, S., Lane, H.C., 2020. Remdesivir for the Treatment of Covid-19 — Final Report. New England Journal of Medicine 383, 1813–1826. https://doi.org/10.1056/nejmoa2007764

Biron, B.M., Chung, C.-S., O'brien, X.M., Chen, Y., Reichner, J.S., Ayala, A., 2016. E-Mail Cl-Amidine Prevents Histone 3 Citrullination and Neutrophil Extracellular Trap Formation, and Improves Survival in a Murine Sepsis Model. https://doi.org/10.1159/000448808

Casanova, V., Sousa, F.H., Shakamuri, P., Svoboda, P., Buch, C., D'Acremont, M., Christophorou, M.A., Pohl, J., Stevens, C., Barlow, P.G., 2020. Citrullination Alters the Antiviral and Immunomodulatory Activities of the Human Cathelicidin LL-37 During Rhinovirus Infection. Frontiers in Immunology 11. https://doi.org/10.3389/fimmu.2020.00085

Chumanevich, A.A., Causey, C.P., Knuckley, B.A., Jones, J.E., Poudyal, D., Chumanevich, A.P., Davis, T., Matesic, L.E., Thompson, P.R., Hofseth, L.J., 2011. Suppression of colitis in mice by Cl-amidine: a novel peptidylarginine deiminase inhibitor. Am J Physiol Gastrointest Liver Physiol 300, G929-938. https://doi.org/10.1152/ajpgi.00435.2010

Conforti, A., Marra, E., Palombo, F., Roscilli, G., Ravà, M., Fumagalli, V., Muzi, A., Maffei, M., Luberto, L., Lione, L., Salvatori, E., Compagnone, M., Pinto, E., Pavoni, E., Bucci, F., Vitagliano, G., Stoppoloni, D., Pacello, M.L., Cappelletti, M., Ferrara, F.F., D'Acunto, E., Chiarini, V., Arriga, R., Nyska, A., Di Lucia, P., Marotta, D., Bono, E., Giustini, L., Sala, E., Perucchini, C., Paterson, J., Ryan, K.A., Challis, A.-R., Matusali, G., Colavita, F., Caselli, G., Criscuolo, E., Clementi, N., Mancini, N., Groß, R., Seidel, A., Wettstein, L., Münch, J., Donnici, L., Conti, M., De Francesco, R., Kuka, M., Ciliberto, G., Castilletti, C., Capobianchi, M.R., Ippolito, G., Guidotti, L.G., Rovati, L., Iannacone, M., Aurisicchio, L., 2021. COVID-eVax, an electroporated DNA vaccine candidate encoding the SARS-CoV-2 RBD, elicits protective responses in animal models. Mol Ther S1525-0016(21)00466–4. https://doi.org/10.1016/j.ymthe.2021.09.011

De Wit, E., Van Doremalen, N., Falzarano, D., Munster, V.J., 2016. SARS and MERS: Recent insights into emerging coronaviruses. Nature Reviews Microbiology. https://doi.org/10.1038/nrmicro.2016.81

Delorey, T.M., Ziegler, C.G.K., Heimberg, G., Normand, R., Yang, Y., Segerstolpe, Å., Abbondanza, D., Fleming, S.J., Subramanian, A., Montoro, D.T., Jagadeesh, K.A., Dey, K.K., Sen, P., Slyper, M., Pita-Juárez, Y.H., Phillips, D., Biermann, J., Bloom-Ackermann, Z., Barkas, N., Ganna, A., Gomez, J., Melms, J.C., Katsyv, I., Normandin, E., Naderi, P., Popov, Y.V., Raju, S.S., Niezen, S., Tsai, L.T.-Y., Siddle, K.J., Sud, M., Tran, V.M., Vellarikkal, S.K., Wang, Y., Amir-Zilberstein, L., Atri, D.S., Beechem, J., Brook, O.R., Chen, J., Divakar, P., Dorceus, P., Engreitz, J.M., Essene, A., Fitzgerald, D.M., Fropf, R., Gazal, S., Gould, J., Grzyb, J., Harvey, T., Hecht, J., Hether, T., Jané-Valbuena, J., Leney-Greene, M., Ma, H., McCabe, C., McLoughlin, D.E., Miller, E.M., Muus, C., Niemi, M., Padera, R., Pan, L., Pant, D., Pe'er, C., Pfiffner-Borges, J., Pinto, C.J., Plaisted, J., Reeves, J., Ross, M., Rudy, M., Rueckert, E.H., Siciliano, M., Sturm, A., Todres, E., Waghray, A., Warren, S., Zhang, S., Zollinger, D.R., Cosimi, L., Gupta, R.M., Hacohen, N., Hibshoosh, H., Hide, W., Price, A.L., Rajagopal, J., Tata, P.R., Riedel, S., Szabo, G., Tickle, T.L., Ellinor, P.T., Hung, D., Sabeti, P.C., Novak, R., Rogers, R., Ingber, D.E., Jiang, Z.G., Juric, D., Babadi, M., Farhi, S.L., Izar, B., Stone, J.R., Vlachos, I.S., Solomon, I.H., Ashenberg, O., Porter, C.B.M., Li, B., Shalek, A.K., Villani, A.-C., Rozenblatt-Rosen, O., Regev, A., 2021. COVID-19 tissue atlases reveal SARS-CoV-2 pathology and cellular targets. Nature 595, 107–113. https://doi.org/10.1038/s41586-021-03570-8

Dittmar, M., Lee, J.S., Whig, K., Segrist, E., Li, M., Kamalia, B., Castellana, L., Ayyanathan, K., Cardenas-Diaz, F.L., Morrisey, E.E., Truitt, R., Yang, W., Jurado, K., Samby, K., Ramage, H., Schultz, D.C., Cherry, S., 2021. Drug repurposing screens reveal cell-type-specific entry pathways and FDA-approved drugs active against SARS-Cov-2. Cell Reports 35, 108959. https://doi.org/10.1016/j.celrep.2021.108959

Fan, Y., Zhao, K., Shi, Z.L., Zhou, P., 2019. Bat coronaviruses in China. Viruses. https://doi.org/10.3390/v11030210

Fischer, W., Eron, J.J., Holman, W., Cohen, M.S., Fang, L., Szewczyk, L.J., Sheahan, T.P., Baric, R., Mollan, K.R., Wolfe, C.R., Duke, E.R., Azizad, M.M., Borroto-Esoda, K., Wohl, D.A., Loftis, A.J., Alabanza, P., Lipansky, F., Painter, W.P., 2021. Molnupiravir, an Oral Antiviral Treatment for COVID-19. https://doi.org/10.1101/2021.06.17.21258639

Ghosh, A.K., Miller, H., Knox, K., Kundu, M., Henrickson, K.J., Arav-Boger, R., 2021. Inhibition of Human Coronaviruses by Antimalarial Peroxides. ACS Infectious Diseases. https://doi.org/10.1021/acsinfecdis.1c00053

Griffante, G., Gugliesi, F., Pasquero, S., Dell'Oste, V., Biolatti, M., Salinger, A.J., Mondal, S., Thompson, P.R., Weerapana, E., Lebbink, R.J., Soppe, J.A., Stamminger, T., Girault, V., Pichlmair, A., Oroszlán, G., Coen, D.M., De Andrea, M., Landolfo, S., 2021. Human cytomegalovirus-induced host protein citrullination is crucial for viral replication. Nature Communications 12, 1–14. https://doi.org/10.1038/s41467-021-24178-6

Hartenian, E., Nandakumar, D., Lari, A., Ly, M., Tucker, J.M., Glaunsinger, B.A., 2020. The molecular virology of coronaviruses. Journal of Biological Chemistry 295, 12910–12934. https://doi.org/10.1074/jbc.REV120.013930

Hsu, J., 2020. Covid-19: What now for remdesivir? BMJ 371, m4457. https://doi.org/10.1136/bmj.m4457

Knight, J.S., Subramanian, V., O'Dell, A.A., Yalavarthi, S., Zhao, W., Smith, C.K., Hodgin, J.B., Thompson, P.R., Kaplan, M.J., 2015. Peptidylarginine deiminase inhibition disrupts NET

formation and protects against kidney, skin and vascular disease in lupus-prone MRL/ *lpr* mice. Annals of the Rheumatic Diseases 74, 2199–2206. https://doi.org/10.1136/annrheumdis-2014-205365

Ksiazek, T.G., Erdman, D., Goldsmith, C.S., Zaki, S.R., Peret, T., Emery, S., Tong, S., Urbani, C., Comer, J.A., Lim, W., Rollin, P.E., Dowell, S.F., Ling, A.-E., Humphrey, C.D., Shieh, W.-J., Guarner, J., Paddock, C.D., Rota, P., Fields, B., DeRisi, J., Yang, J.-Y., Cox, N., Hughes, J.M., LeDuc, J.W., Bellini, W.J., Anderson, L.J., 2003. A Novel Coronavirus Associated with Severe Acute Respiratory Syndrome. New England Journal of Medicine 348, 1953–1966. https://doi.org/10.1056/nejmoa030781

Leao, J.C., Gusmao, T.P. de L., Zarzar, A.M., Leao Filho, J.C., Barkokebas Santos de Faria, A., Morais Silva, I.H., Gueiros, L.A.M., Robinson, N.A., Porter, S., Carvalho, A. de A.T., 2020. Coronaviridae—Old friends, new enemy! Oral Diseases 0–3. https://doi.org/10.1111/odi.13447

Ledet, M.M., Anderson, R., Harman, R., Muth, A., Thompson, P.R., Coonrod, S.A., Van de Walle, G.R., 2018. BB-Cl-Amidine as a novel therapeutic for canine and feline mammary cancer via activation of the endoplasmic reticulum stress pathway. BMC Cancer 18, 1–13. https://doi.org/10.1186/s12885-018-4323-8

Lee, C.-Y., Lowen, A.C., 2021. Animal models for SARS-CoV-2. Current Opinion in Virology 48, 73–81. https://doi.org/10.1016/j.coviro.2021.03.009

Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., Wang, W., Song, H., Huang, B., Zhu, N., Bi, Y., Ma, X., Zhan, F., Wang, L., Hu, T., Zhou, H., Hu, Z., Zhou, W., Zhao, L., Chen, J., Meng, Y., Wang, J., Lin, Y., Yuan, J., Xie, Z., Ma, J., Liu, W.J., Wang, D., Xu, W., Holmes, E.C., Gao, G.F., Wu, G., Chen, W., Shi, W., Tan, W., 2020. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. The Lancet 395, 565–574. https://doi.org/10.1016/S0140-6736(20)30251-8

Marcello, A., Civra, A., Milan Bonotto, R., Nascimento Alves, L., Rajasekharan, S., Giacobone, C., Caccia, C., Cavalli, R., Adami, M., Brambilla, P., Lembo, D., Poli, G., Leoni, V., 2020. The cholesterol metabolite 27-hydroxycholesterol inhibits SARS-CoV-2 and is markedly decreased in COVID-19 patients. Redox Biology 36, 101682. https://doi.org/10.1016/j.redox.2020.101682

Milani, M., Donalisio, M., Bonotto, R.M., Schneider, E., Arduino, I., Boni, F., Lembo, D., Marcello, A., Mastrangelo, E., 2021. Combined in silico and in vitro approaches identified the antipsychotic drug lurasidone and the antiviral drug elbasvir as SARS-CoV2 and HCoV-OC43 inhibitors. Antiviral Research 189, 105055. https://doi.org/10.1016/j.antiviral.2021.105055

Milewska, A., Kaminski, K., Ciejka, J., Kosowicz, K., Zeglen, S., Wojarski, J., Nowakowska, M., Szczubiałka, K., Pyrc, K., 2016. HTCC: Broad range inhibitor of coronavirus entry. PLoS ONE 11, 1–17. https://doi.org/10.1371/journal.pone.0156552

Mondal, S., Thompson, P.R., 2019. Protein Arginine Deiminases (PADs): Biochemistry and Chemical Biology of Protein Citrullination. Accounts of Chemical Research 52, 818–832. https://doi.org/10.1021/acs.accounts.9b00024

Ogando, N.S., Dalebout, T.J., Zevenhoven-Dobbe, J.C., Limpens, R.W.A.L., van der Meer, Y., Caly, L., Druce, J., de Vries, J.J.C., Kikkert, M., Bárcena, M., Sidorov, I., Snijder, E.J., 2020. SARS-coronavirus-2 replication in Vero E6 cells: replication kinetics, rapid adaptation and cytopathology. J Gen Virol 101, 925–940. https://doi.org/10.1099/jgv.0.001453

Paules, C.I., Marston, H.D., Fauci, A.S., 2020. Coronavirus Infections-More Than Just the Common Cold. JAMA - Journal of the American Medical Association. https://doi.org/10.1001/jama.2020.0757

Pratesi, F., Tommasi, C., Anzilotti, C., Chimenti, D., Migliorini, P., 2006. Deiminated Epstein-Barr virus nuclear antigen 1 is a target of anti-citrullinated protein antibodies in rheumatoid arthritis. Arthritis and Rheumatism 54, 733–741. https://doi.org/10.1002/art.21629

Pratesi, F., Tommasi, C., Anzilotti, C., Puxeddu, I., Sardano, E., Di Colo, G., Migliorini, P., 2011. Antibodies to a new viral citrullinated peptide, VCP2: fine specificity and correlation with anti-cyclic citrullinated peptide (CCP) and anti-VCP1 antibodies. Clinical & Experimental Immunology 164, 337–345. https://doi.org/10.1111/j.1365-2249.2011.04378.x

Ravikumar, B., Aittokallio, T., 2018. Improving the efficacy-safety balance of polypharmacology in multi-target drug discovery. Expert Opin Drug Discov 13, 179–192. https://doi.org/10.1080/17460441.2018.1413089

Sokolove, J., Brennan, M.J., Sharpe, O., Lahey, L.J., Kao, A.H., Krishnan, E., Edmundowicz, D., Lepus, C.M., Wasko, M.C., Robinson, W.H., 2013. Brief report: citrullination within the atherosclerotic plaque: a potential target for the anti-citrullinated protein antibody response in rheumatoid arthritis. Arthritis Rheum 65, 1719–1724. https://doi.org/10.1002/art.37961

Su, S., Wong, G., Shi, W., Liu, J., Lai, A.C.K., Zhou, J., Liu, W., Bi, Y., Gao, G.F., 2016. Epidemiology, Genetic Recombination, and Pathogenesis of Coronaviruses. https://doi.org/10.1016/j.tim.2016.03.003

Trier, N.H., Holm, B.E., Heiden, J., Slot, O., Locht, H., Lindegaard, H., Svendsen, A., Nielsen, C.T., Jacobsen, S., Theander, E., Houen, G., 2018. Antibodies to a strain-specific citrullinated Epstein-Barr virus peptide diagnoses rheumatoid arthritis. Scientific Reports 8, 3684. https://doi.org/10.1038/s41598-018-22058-6

van Venrooij, W.J., van Beers, J.J.B.C., Pruijn, G.J.M., 2011. Anti-CCP antibodies: the past, the present and the future. Nat Rev Rheumatol 7, 391–398. https://doi.org/10.1038/nrrheum.2011.76

Vossenaar, E.R., Zendman, A.J.W., Van Venrooij, W.J., Pruijn, G.J.M., 2003. PAD, a growing family of citrullinating enzymes: Genes, features and involvement in disease. BioEssays 25, 1106–1118. https://doi.org/10.1002/bies.10357

Warren, T.K., Jordan, R., Lo, M.K., Ray, A.S., Mackman, R.L., Soloveva, V., Siegel, D., Perron, M., Bannister, R., Hui, H.C., Larson, N., Strickley, R., Wells, J., Stuthman, K.S., Van Tongeren, S.A., Garza, N.L., Donnelly, G., Shurtleff, A.C., Retterer, C.J., Gharaibeh, D., Zamani, R., Kenny, T., Eaton, B.P., Grimes, E., Welch, L.S., Gomba, L., Wilhelmsen, C.L., Nichols, D.K., Nuss, J.E., Nagle, E.R., Kugelman, J.R., Palacios, G., Doerffler, E., Neville, S., Carra, E., Clarke, M.O., Zhang, L., Lew, W., Ross, B., Wang, Q., Chun, K., Wolfe, L., Babusis, D., Park, Y., Stray, K.M., Trancheva, I., Feng, J.Y., Barauskas, O., Xu, Y., Wong, P., Braun, M.R., Flint, M., McMullan, L.K., Chen, S.S., Fearns, R., Swaminathan, S., Mayers, D.L., Spiropoulou, C.F., Lee, W.A., Nichol, S.T., Cihlar, T., Bavari, S., 2016. Therapeutic efficacy of the small molecule GS-5734 against Ebola virus in rhesus monkeys. Nature 531, 381–385. https://doi.org/10.1038/nature17180

Weiss, S.R., Navas-Martin, S., 2005. Coronavirus Pathogenesis and the Emerging Pathogen Severe Acute Respiratory Syndrome Coronavirus. MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS 69, 635–664. https://doi.org/10.1128/MMBR.69.4.635-664.2005

Willis, V.C., Banda, N.K., Cordova, K.N., Chandra, P.E., Robinson, W.H., Cooper, D.C., Lugo, D., Mehta, G., Taylor, S., Tak, P.P., Prinjha, R.K., Lewis, H.D., Holers, V.M., 2017. Protein arginine deiminase 4 inhibition is sufficient for the amelioration of collagen-induced arthritis. Clin Exp Immunol 188, 263–274. https://doi.org/10.1111/cei.12932

Willis, V.C., Gizinski, A.M., Banda, N.K., Causey, C.P., Knuckley, B., Cordova, K.N., Luo, Y., Levitt, B., Glogowska, M., Chandra, P., Kulik, L., Robinson, W.H., Arend, W.P., Thompson, P.R., Holers, V.M., 2011. N-α-Benzoyl-N5-(2-Chloro-1-Iminoethyl)-l-Ornithine Amide, a Protein Arginine Deiminase Inhibitor, Reduces the Severity of Murine Collagen-Induced Arthritis. The Journal of Immunology 186, 4396–4404. https://doi.org/10.4049/jimmunol.1001620

Wing, P.A.C., Keeley, T.P., Zhuang, X., Lee, J.Y., Prange-Barczynska, M., Tsukuda, S., Morgan, S.B., Harding, A.C., Argles, I.L.A., Kurlekar, S., Noerenberg, M., Thompson, C.P., Huang, K.Y.A., Balfe, P., Watashi, K., Castello, A., Hinks, T.S.C., James, W., Ratcliffe, P.J., Davis, I., Hodson, E.J., Bishop, T., McKeating, J.A., 2021. Hypoxic and pharmacological activation of HIF inhibits SARS-CoV-2 infection of lung epithelial cells. Cell Reports 35, 109020. https://doi.org/10.1016/j.celrep.2021.109020

Witalisom, E., Thompson, R., Hofseth, L., 2002. Protein Arginine Deiminases and Associated Cirtullination. Anuario musical: Revista de musicolog{í}a del CSIC 16, 199–227.

Wu, F., Zhao, S., Yu, B., Chen, Y.M., Wang, W., Song, Z.G., Hu, Y., Tao, Z.W., Tian, J.H., Pei, Y.Y., Yuan, M.L., Zhang, Y.L., Dai, F.H., Liu, Y., Wang, Q.M., Zheng, J.J., Xu, L., Holmes, E.C., Zhang, Y.Z., 2020. A new coronavirus associated with human respiratory disease in China. Nature 579, 265–269. https://doi.org/10.1038/s41586-020-2008-3

Yang, L., Tan, D., Piao, H., 2016. Myelin Basic Protein Citrullination in Multiple Sclerosis: A Potential Therapeutic Target for the Pathology. Neurochemical Research 41, 1845–1856. https://doi.org/10.1007/s11064-016-1920-2

Yuzhalin, A.E., 2019. Citrullination in Cancer. Cancer Res 79, 1274–1284. https://doi.org/10.1158/0008-5472.CAN-18-2797

Zaki, A.M., van Boheemen, S., Bestebroer, T.M., Osterhaus, A.D.M.E., Fouchier, R.A.M., 2012. Isolation of a Novel Coronavirus from a Man with Pneumonia in Saudi Arabia. New England Journal of Medicine 367, 1814–1820. https://doi.org/10.1056/nejmoa1211721

Zhao, T., Pan, B., Alam, H.B., Liu, B., Bronson, R.T., Deng, Q., Wu, E., Li, Y., 2016. Protective effect of Cl-amidine against CLP-induced lethal septic shock in mice. Sci Rep 6, 36696. https://doi.org/10.1038/srep36696

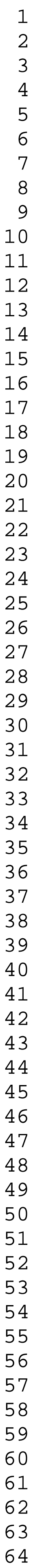


Fig. 1. The pan-PAD inhibitors Cl-A and BB-Cl hamper HCoV-OC43 replication in MRC-5 cells. (A) Protein lysates from MRC-5 cells infected with HCoV-OC43 (MOI 1) at 48 and 72 h post infection (hpi) or from uninfected control cells (Mock) were exposed to Rh-PG probe (top panel) and subjected to gel electrophoresis to detect citrullinated proteins. Viral protein expression (OC43 NP) and equal loading (actin) were assessed by Western blot analysis (bottom panels). One

representative gel of three independent experiments is shown. (B, C) Dose-response curves of the cell-permeable pan-PAD inhibitors Cl-A (A) and BB-Cl (B). MRC-5 cells were infected with HCoV-OC43 (MOI 0.1) and treated with increasing amounts of the drugs, which were given 1 h prior to virus adsorption and kept throughout the whole experiment. After 72 hpi, the viral load was determined by RT-PCR and expressed as percentage relative to the untreated control. Values are expressed as mean ± SEM of three independent experiments. (D) MRC-5 cells were pre-treated with Cl-A (100 μM) or BB-Cl (2.5 μM) for 1 h and then infected with HCoV-OC43 (MOI 0.1). After 72 hpi, viral replication was assessed by RT-PCR on cell-associated viral RNA, previously extracted from infected cells, or on the supernatants collected from infected cells. Values are expressed as mean ± SEM of three independent experiments. (E) MRC-5 cells were infected with HCoV-OC43 (MOI 0.1) and then treated with Cl-A (100 μM) or BB-Cl (2.5 μM) as described in B. Protein lysates were prepared at 48 hpi and exposed to Rh-PG probe (top panel). Viral protein expression (OC43 NP) and equal loading (actin) were assessed by Western blot analysis (one representative experiment out of three is reported, bottom panels) and quantified by densitometric analysis (right panel). (F) Viral productions were collected at 72 hpi and analyzed by plaque-forming assay. Values are expressed as mean ± SEM of three independent experiments. P-value < 0.05 (*), < 0.01 (**), < 0.001 (***) and < 0.0001 (****).

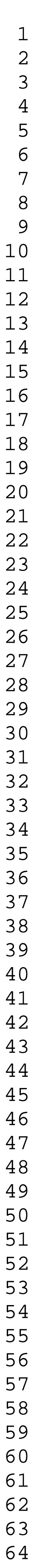


Fig. 2. Effect of specific PAD2 and PAD4 inhibitors on HCoV-OC43 replication in MRC-5 cells. (A) Western blot analysis of protein lysates from uninfected (mock) or infected MRC-5 cells using antibodies against PAD1, PAD2, PAD3, PAD4, PAD6, or β-actin. The blot shown is representative

of three independent experiments. (B-C) Dose-response curves of the PAD2 (AFM30a) and PAD4 (GSK199) inhibitors. MRC-5s were infected with HCoV-OC43 (MOI 0.1) and then treated with increasing concentrations of the aforementioned drugs, as described in the legend to Fig 1. After 72 hpi, the viral load was determined by RT-PCR and expressed as a percentage relative to the untreated control. Values are expressed as mean ± SEM of three independent experiments. (D) MRC-5 cells were pre-treated with AFM30a (20 µM) or GSK199 (20 µM) for 1 h. Subsequently, cells were infected with HCoV-OC43 (MOI 0.1). The extent of HCoV-OC43 replication was then assessed by RT-PCR of viral RNA contained in cellular lysates (cell associated) or supernatants (cell-free) at 72 hpi. Values are expressed as mean ± SEM of three independent experiments. (E, F) MRC-5 cells were infected with HCoV-OC43 (MOI 0.1) and then treated with AFM30a or GSK199, as described in D. Protein lysates were prepared at 48 hpi and viral protein expression (OC43 NP) and equal loading (β-actin) were assessed by Western blot analysis (one representative experiment out of three is reported in E, left panel) and quantified by densitometric analysis (E, right panel). Viral productions were collected at 72 hpi and analyzed by plaque assay (F). Values are expressed as mean ± SEM of three independent experiments. *P*-value < 0.05 (*), < 0.01 (**), < 0.001 (***) and < 0.0001 (****).

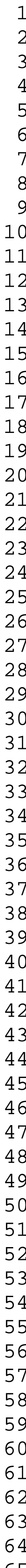
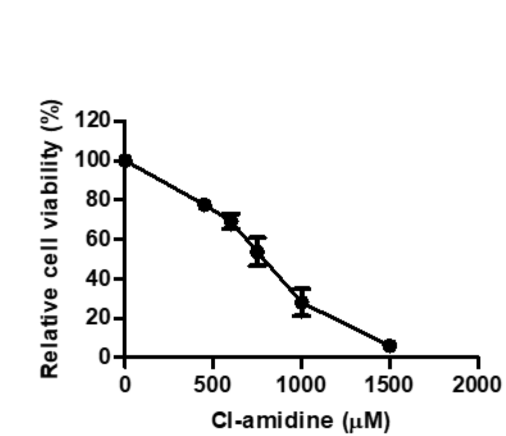


Fig. 3. The pan-PAD inhibitors Cl-Amidine and BB-Cl-Amidine block β-coronavirus replication in Vero-E6 cells. (A, B) Vero-E6 cells were infected with HCoV-OC43 (MOI 0.1) and then treated with increasing concentrations of Cl-A (A) or BB-Cl (B), which were given 1 h prior to virus adsorption and kept throughout the whole experiment. After 72 hpi, the viral load was determined by RT-PCR and expressed as a percentage relative to the untreated control. Values are expressed as mean ± SEM of three independent experiments. (C) Vero-E6 cells were treated with Cl-

A (300 μM) or with equal volumes of DMSO 1 h before infection and for the entire duration of the infection, and infected with HCoV-OC43 at a MOI of 1. Protein lysates were prepared at 48 hpi and subjected to Rh-PG (top panel) and Western blot (bottom panels) analyses. One representative blot is shown of three independent experiments. (D, E) Vero-E6 cells were infected with SARS-CoV-2 (MOI 0.1) and then treated as described in A. After 72 hpi, the viral load was determined by RT-PCR and expressed as the percentage relative to untreated control. Values are expressed as mean ± SEM of three independent experiments. (F) Vero-E6 cells were infected with SARS-CoV-2 (MOI 1) and then treated as described in C. Protein lysates were prepared at 48 hpi and subjected to Rh-PG (top panel) and Western blot (bottom panels) analyses. The blot shown is representative of three independent experiments. (G) Vero-E6 cells were infected with SARS-CoV-2 (MOI 0.1) and then treated as described in C. Viral productions were collected at 72 hpi and analyzed by plaque assay. Values are expressed as mean ± SEM of three independent experiments. *P*-value < 0.05 (*), < 0.01 (**), < 0.001 (***) and < 0.0001 (****).

Supplementary

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65



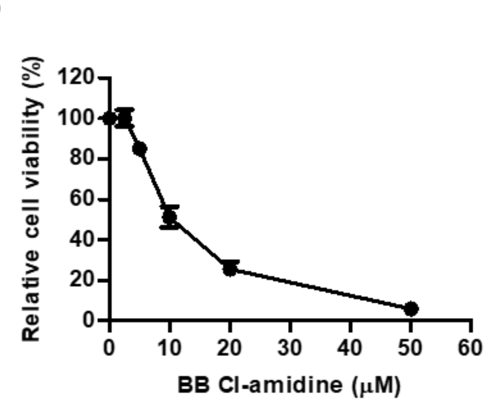
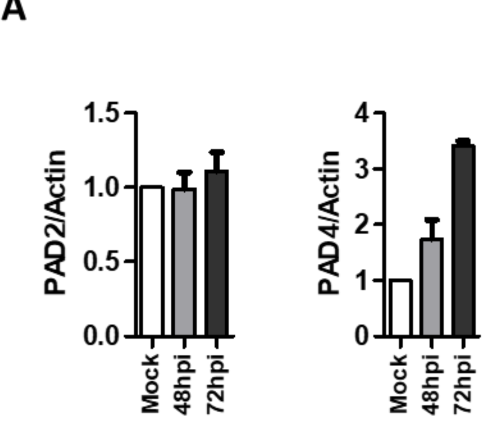


Figure S1



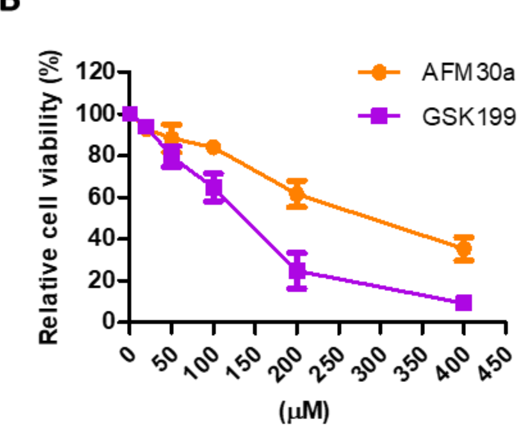
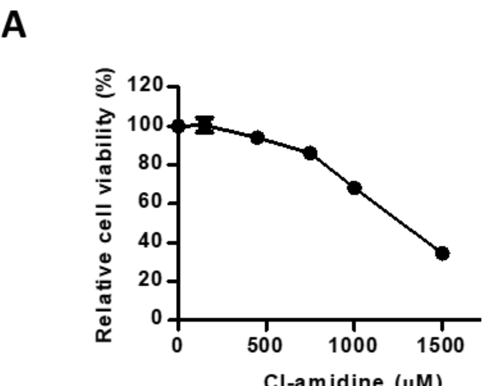


Figure S2



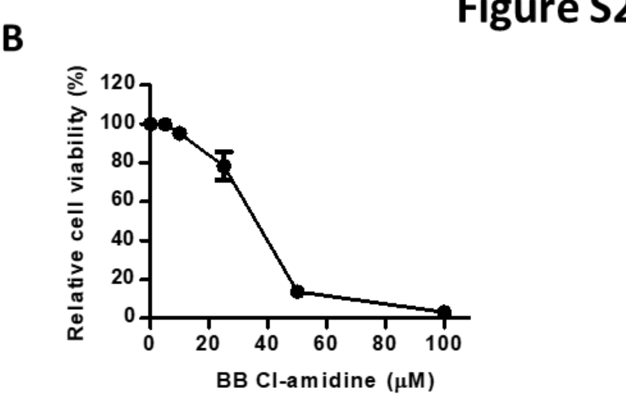


Figure S3

Fig. S1. (A, B) MTT assay. Uninfected MRC-5 cells were treated with the indicated concentrations of Cl-amidine and BB-Cl-amidine or DMSO alone for 72 h and subjected to MTT assay. Values are expressed as mean ± SEM of three independent experiments.

Fig. S2. (A, B) Densitometric analysis. **C)** MTT assay. Uninfected MRC-5 cells were treated MRC-5 cells were treated with the indicated concentrations of AFM30a or GSK199 or DMSO alone for 72 h. Values are expressed as mean ± SEM of three independent experiments.

Fig. S3 (A, B) The MTT assay was performed in uninfected Vero-E6 cells using different concentrations of drugs. MRC-5 cells were treated with different concentrations of Cl-A (A) or BB-Cl (B) for 72 h. Values are expressed as mean ± SEM of three independent experiments.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Table 1

CC_{50}, IC_{50}, and SI of PAD inhibitors against beta-CoVs

cell/virus	compound	[µM]		
		IC50	CC50	SI
MRC-5/OC43	Cl-Amidine	34.80	949.14	27.30
	BB-Cl-Amidine	0.53	10.12	18.62
	GSK199	0.60	133.08	224.94
	AFM30a	> 20	320.92	> 16
Vero-E6/OC43	Cl-Amidine	44.15	> 1000	> 22
	BB-Cl-Amidine	10.68	33.06	3.10
Vero-E6/SARS-CoV-2	Cl-Amidine	95.17	> 1000	> 10
	BB-Cl-Amidine	17.78	33.06	1.86

Click here to access/download
Supplementary Material
Supplementary.pdf

ARTICLE

https://doi.org/10.1038/s41467-021-24178-6 OPEN

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

Gloria Griffante[1,2], Francesca Gugliesi[1], Selina Pasquero[1], Valentina Dell'Oste[1], Matteo Biolatti[1], Ari J. Salinger[3,4], Santanu Mondal[3], Paul R. Thompson[3], Eranthie Weerapana[4], Robert J. Lebbink[5], Jasper A. Soppe[5], Thomas Stamminger[6], Virginie Girault[7], Andreas Pichlmair[7], Gábor Oroszlán[8], Donald M. Coen[8], Marco De Andrea[1,9]✉ & Santo Landolfo[1]✉

Citrullination is the conversion of arginine-to-citrulline by protein arginine deiminases (PADs), whose dysregulation is implicated in the pathogenesis of various types of cancers and autoimmune diseases. Consistent with the ability of human cytomegalovirus (HCMV) to induce post-translational modifications of cellular proteins to gain a survival advantage, we show that HCMV infection of primary human fibroblasts triggers PAD-mediated citrullination of several host proteins, and that this activity promotes viral fitness. Citrullinome analysis reveals significant changes in deimination levels of both cellular and viral proteins, with interferon (IFN)-inducible protein IFIT1 being among the most heavily deiminated one. As genetic depletion of IFIT1 strongly enhances HCMV growth, and in vitro IFIT1 citrullination impairs its ability to bind to 5′-ppp-RNA, we propose that viral-induced IFIT1 citrullination is a mechanism of HCMV evasion from host antiviral resistance. Overall, our findings point to a crucial role of citrullination in subverting cellular responses to viral infection.

[1] Department of Public Health and Pediatric Sciences, University of Turin, Turin, Italy. [2] Department of Translational Medicine, University of Piemonte Orientale, Novara, Italy. [3] Department of Biochemistry and Molecular Pharmacology, UMass Medical School, Worcester, MA, USA. [4] Department of Chemistry, Boston College, Chestnut Hill, MA, USA. [5] Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands. [6] Institute of Virology, Ulm University Medical Center, Ulm, Germany. [7] Institute of Virology, Technical University of Munich, Munich, Germany. [8] Department of Biological Chemistry & Molecular Pharmacology, Blavatnik Institute, Harvard Medical School, Boston, MA, USA. [9] CAAD Center for Translational Research on Autoimmune and Allergic Disease, University of Piemonte Orientale, Novara, Italy. ✉email: marco.deandrea@unito.it; santo.landolfo@unito.it

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 autoimmune diseases and degenerative disorders, such as atherosclerosis, 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].

A post-translational modification (PTM) that is increasingly recognized to play an essential role in immune-related diseases is citrullination, also called deimination, a process where the guanidinium group of arginine is hydrolyzed to form citrulline, a non-genetically encoded amino acid[10]. 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[11]. Although aberrant citrullination has been detected in several inflammatory conditions, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Alzheimer's disease (AD), multiple sclerosis (MS), Parkinson's disease, cancer, and atherosclerosis, suggesting that it may play a pathogenic role in inflammation-related diseases[12–19], a direct correlation between citrullination and viral infections has only recently emerged. In particular, human rhinovirus (HRV)-induced citrullination of LL37 was found to impair the antiviral activity of this host defense protein[20]. Consistent with the role of citrullination in viral infections, other studies have shown how artificially citrullinated Epstein–Barr virus (EBV) proteins could be specifically recognized by RA sera[21–23]. Importantly, a recent in silico analysis of transcriptome datasets from lung biopsies of SARS-CoV-2-infected patients has proposed a putative role of PADs in COVID-19[24].

In this study, we unveil a signature of HCMV infection based on PAD-mediated citrullination of multiple cellular proteins to disrupt host -defense mechanisms.

Results

HCMV infection induces protein citrullination. To begin to characterize protein citrullination during HCMV infection, we performed immunoblot analysis of protein lysates obtained from human foreskin fibroblasts (HFFs) infected with the HCMV strain Merlin at different time points, using the citrulline-specific probe rhodamine–phenylglyoxal (Rh–PG)[25]. We observed an increase in total protein citrullination in lysates from HCMV-infected HFFs at 48 h and, albeit to a lesser extent, at 96 h post infection (hpi) compared to uninfected control cells (mock) (Fig. 1a).

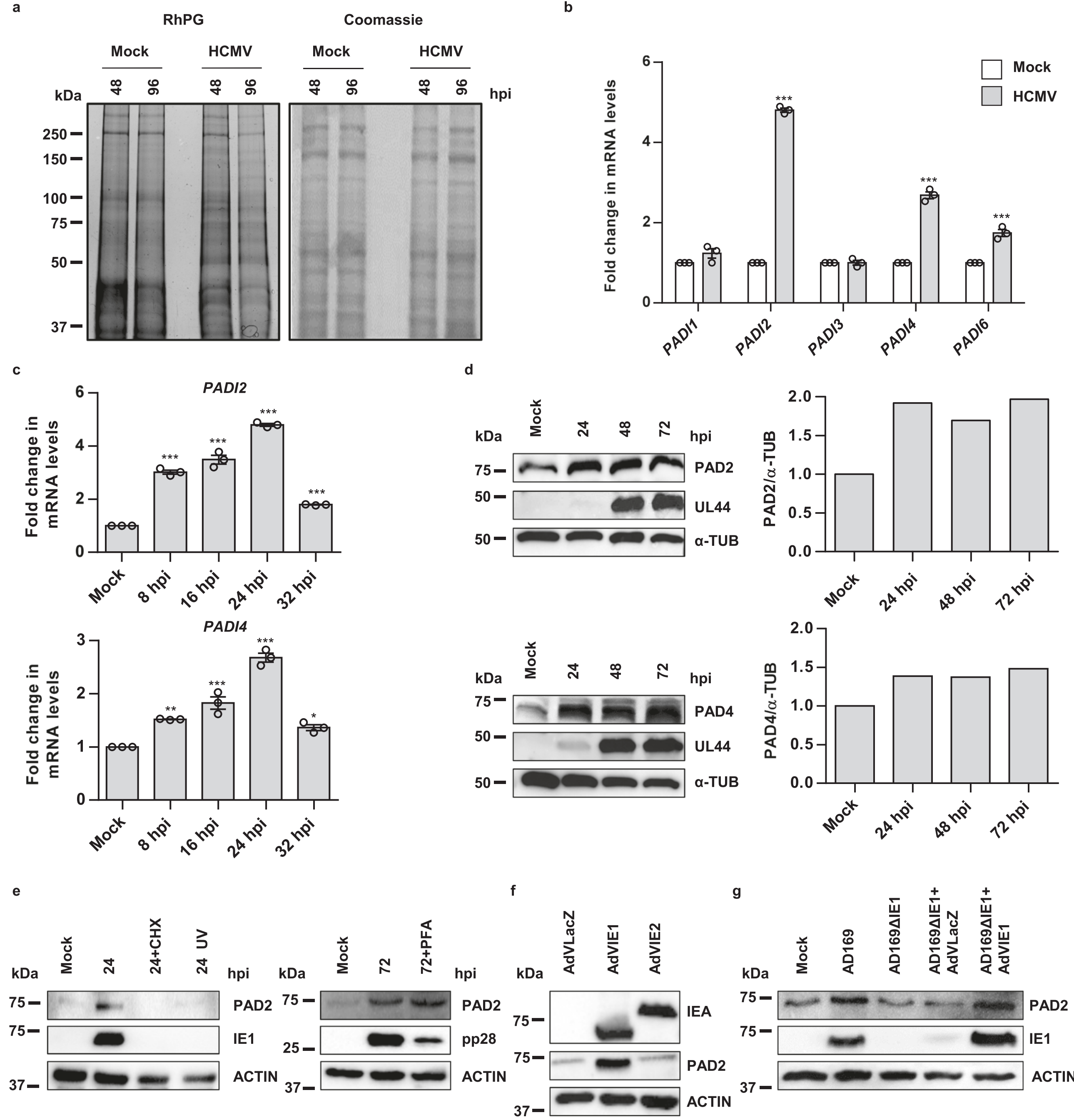
We next sought to determine whether PADs played a role in enhanced citrullination by HCMV. RT-qPCR analysis revealed that *PADI2*, *4*, and *6* genes were all expressed at significantly higher levels in HCMV-infected HFFs at 24 hpi compared to mock-infected controls (i.e., ~4.8-, ~2.7, and ~1.7-fold, respectively) (Fig. 1b). In particular, both *PADI2* and *4* genes were upregulated at 8 hpi, reaching a peak 24 hpi and decreasing afterward (Fig. 1c). In contrast to their mRNA expression levels, PAD2 and 4 protein levels were already increased at 24 hpi and remained elevated for up to 72 hpi (Fig. 1d), suggesting that transcription and translation of PADs are differently regulated upon HCMV infection. By contrast, the other PAD isoforms were expressed at very low (PAD3) or undetectable levels (PAD1 and 6) and did not vary following HCMV infection (Fig. 1b and Supplementary Fig. 1a), indicating that PAD2 and 4 are the only PAD enzymes involved in HCMV-induced citrullination.

PAD protein levels are induced by HCMV immediate early 1 (IE1) protein. 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*. Twenty-four hours after 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. 1c, HCMV infection led to a robust induction of the luciferase activity 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 was responsible for the upregulation of PAD2, the most potently induced PAD member following HCMV infection (Fig. 1b, c). 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 expression of viral proteins 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 both 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 (AD169), an effect that was reversed by AdVIE1 co-infection (Fig. 1g)[26]. In line with previous results, induction of citrullination by the AD169 strain was also confirmed using the citrulline-specific probe Rh–PG (Supplementary Fig. 1d).

PAD inhibition blocks HCMV replication. Next, we asked whether citrullination induced by HCMV infection would affect viral replication. To answer this question, we assessed viral plaque formation in HCMV-infected HFFs (MOI 0.1) treated for 1 h prior to infection with increasing concentrations of Cl-amidine (Cl-A) (25–200 μM), a cell-permeable pan-PAD inhibitor[27]. After 7 days of continuous exposure to Cl-A, we observed a dose-dependent downregulation in the number of viral particles in HCMV-infected HFFs, with a complete suppression at 100 μM. Assessment of cell cytotoxicity by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in HFFs treated under the same conditions ruled out the possibility that the antiviral activity was related to the cytotoxic effects



of the drug (Fig. 2a). The half-maximal inhibitory concentration (IC_{50}) of Cl-A was found to be 36 µM (Fig. 2a). To further corroborate these data, we also measured viral DNA synthesis by quantitative real-time PCR (qPCR) in similarly treated cells. Remarkably, at the low concentration of 25 µM, Cl-A treatment reduced the rate of HCMV DNA replication by approximately threefold compared to untreated cells (Fig. 2b). In line with the plaque-forming activity data, treatment with 100 µM Cl-A completely shut down HCMV DNA replication (Fig. 2b). Next, HFFs were treated for 1 h prior to HCMV infection with 100 µM Cl-A, 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-A maintained its antiviral effect even at higher HCMV MOIs (0.5–1). Consistently, this remarkable reduction in the number of viral particles was observed also in HCMV-infected HFFs treated with BB-Cl-amidine (BB-Cl-A)[14], a second-generation pan-PAD inhibitor, at nontoxic concentrations much lower than Cl-A of 1 and 0.5 µM, with a complete suppression at 1 µM (Supplementary Fig. 2a).

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 increasing Cl-A concentrations on the replication of: (i) herpes simplex virus 1 (HSV-1) and 2 (HSV-2), two members of the Herpesviridae family; (ii) a clinical isolate of adenovirus as a prototype of a non-enveloped DNA virus; and (iii) the RNA virus human immunodeficiency virus 1 strain IIIb ($HIV\text{-}1_{IIIb}$). Interestingly, only HSV-1 and -2 displayed impaired viral growth in Cl-A-treated cells (IC_{50} ~66 and 21 µM) (Supplementary Fig. 2b), whereas the replication rates of adenovirus and $HIV\text{-}1_{IIIb}$ were only marginally affected by the

Fig. 1 HCMV infection induces protein citrullination. a Protein lysates from HFFs infected with HCMV strain Merlin (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). One representative gel of three independent experiments is shown. **b** mRNA expression levels of *PADI* isoforms by RT-qPCR 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 ± SEM over mock-infected cells ($n = 3$ independent determinations; *PADI2*, *PADI4*, and *PADI6* $P < 0.001$, two-way ANOVA followed by Bonferroni's post test). **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 ± SEM over mock-infected cells ($n = 3$ independent determinations; *PADI2*: mock vs. 8 hpi $P < 0.001$, mock vs. 16 hpi $P < 0.001$, mock vs. 24 hpi $P < 0.001$, mock vs. 32 hpi $P < 0.001$; *PADI4*: mock vs. 8 hpi $P = 0.00375$, mock vs. 16 hpi $P < 0.001$, mock vs. 24 hpi $P < 0.001$, mock vs. 32 hpi $P = 0.04$, one-way ANOVA followed by Bonferroni's post tests). **d** Western blot analysis of protein lysates from uninfected (mock) or infected HFFs using antibodies against PAD2, PAD4, UL44, or α-tubulin (α-TUB). One representative blot and densitometric analysis shown 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) (left panel), 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 (recognizing IE1-72- and IE2-86 kDa), 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 48 h with AdVIE1, AdVIE2, or AdVLacZ (MOI = 10) using antibodies against IEA (recognizing IE1-72- and IE2-86 kDa), PAD2, or ACTIN. One representative blot of three independent experiments is shown. **g** Western blot analysis of protein lysates from HFFs infected with wild-type AD169 HCMV (AD169) or AD169ΔIE1 (MOI 1 PFU/cell), the latter complemented with AdVLacZ or AdVIE1 (MOI 10 PFU/cell), at 24 hpi or from uninfected HFFs (mock) using antibodies against IEA (recognizing IE1-72- and IE2-86 kDa), PAD2, or ACTIN. One representative blot of three independent experiments is shown. Data are shown as the mean ± SEM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

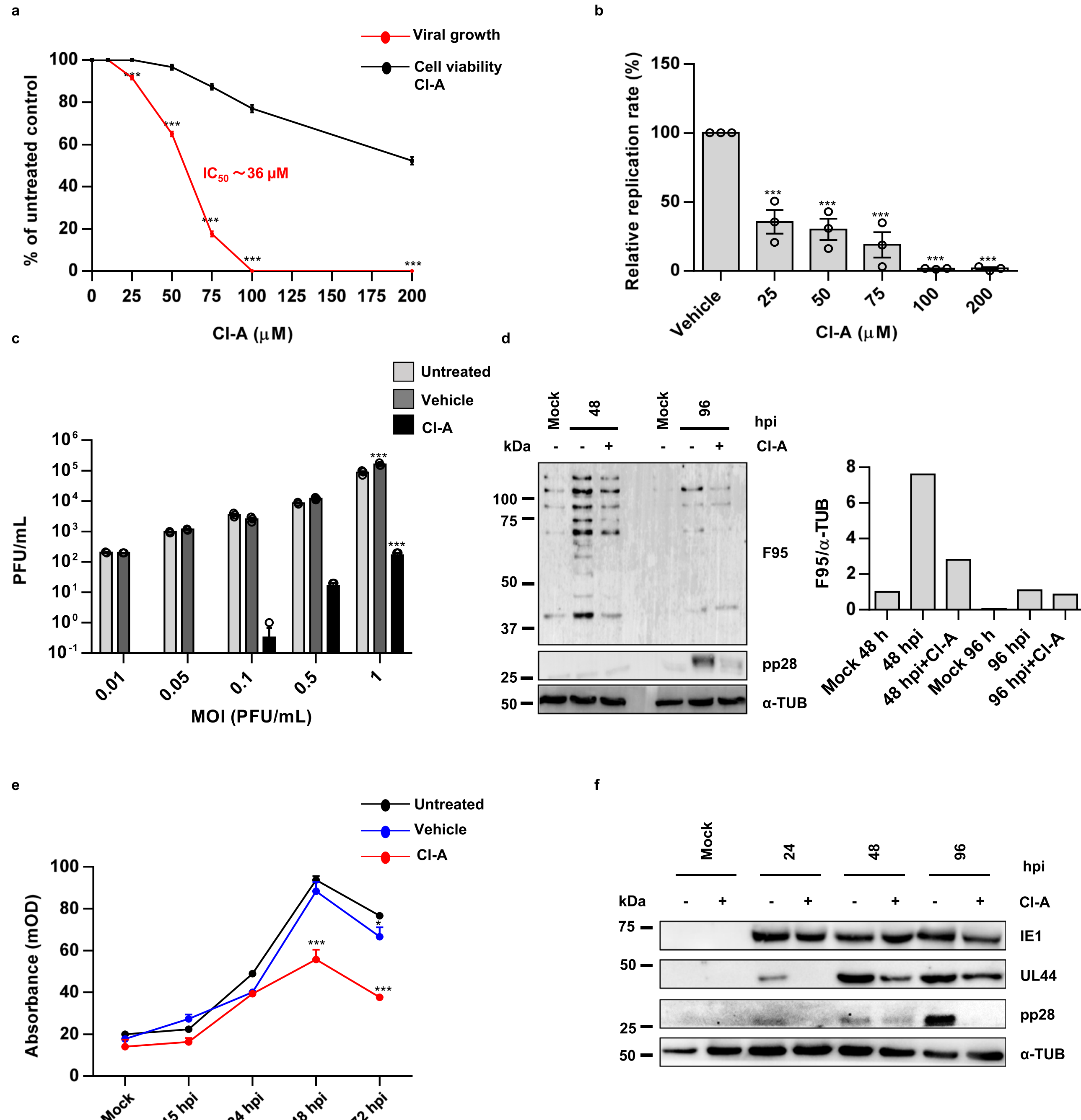
PAD inhibitor (Supplementary Fig. 2c). Of note, the slight reduction in adenovirus replication observed in HFFs cells treated with higher doses of Cl-A was probably due to the cytotoxic effect of the compound, as judged by the MTT assay.

To further demonstrate that Cl-A 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 (Fig. 2d). As expected, in the presence of Cl-A, protein citrullination was efficiently suppressed at either time points (Fig. 2d). Next, PAD enzymatic activity was measured by means of an in vitro antibody-based assay using histone 3 as a 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-A (Supplementary Fig. 1e). Consistent with the kinetics of PAD2 and PAD4 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-A (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-A at various time points post infection were subjected to immunoblotting using antibodies against the corresponding IEA, early (UL44), and late (pp28) proteins. While Cl-A 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-A 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), or PAD4 (PAD4 KO) 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 signals between 50 and 60% (Fig. 3a and Supplementary Fig. 4a). 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 a standard plaque assay and qPCR as described above. As shown in Fig. 3b, c, PAD2 and PAD4 KO cells, respectively, displayed 0.5 and 1 logs of viral load reduction, and ~60 and 70% decrease in viral replication, at an MOI of 0.1. In line with these results, a consistent decrease in UL44 protein expression levels was also detected in infected PAD2, and PAD4 KO cell lines at 48 hpi (Fig. 3a). As expected, depletion of PAD1 (PAD1 KO), 3 (PAD3 KO), or 6 (PAD6 KO) in HFFs did not affect the HCMV replication rates compared to wild-type control cells (Supplementary Fig. 4b, c). Thus, PAD2 and 4 but not PAD1, 3, and 6 sustain the viral cell cycle of HCMV. These results were further confirmed in HFFs transfected with siRNA against PAD2 and PAD4, where a protein knockdown efficiency of ~100 and 70%, respectively (Supplementary Fig. 4d), resulted in an even higher viral reduction of 1 and 2 logs, respectively (Supplementary Fig. 4e). In addition, while siRNA control-transfected HFFs treated with 100 μM Cl-A showed a 1-log reduction in the viral load, no significant differences were observed in PAD2- or PAD4-depleted cells (Supplementary Fig. 4e), indicating no predominant role of one isoform over the other in our working model.

IFN-inducible proteins are the main targets of PAD-mediated citrullination. To identify which proteins (i.e., cellular and/or viral) were deiminated 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 1, 2, and 3), confirming our previous results (Fig. 1a). In addition to detecting numerous citrullinated viral proteins—35 at 48 hpi and 40 proteins at 96 hpi—we noticed an even higher number of



citrullinated host cell proteins—177 at 48 hpi and 122 at 96 hpi. Using PANTHER software, we were able to identify a wide range of citrullinated host proteins falling into various functional classes, among which cytoskeletal proteins, chaperones, oxidoreductase, hydrolase, and nucleic acid binding proteins were more frequently found at both time points of infection (Supplementary Fig. 5a). Of particular interest was the significant citrullination at 48 hpi 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).

To validate these 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, IEA, UL44, and pp65. As shown in Supplementary Fig. 5b, all five proteins were robustly deiminated following infection with HCMV.

To corroborate our finding that IE1 is crucial for PAD induction and subsequent citrullination, we performed an additional citrullinome analysis on uninfected HFFs, and wild-type AD169, or mutant AD169ΔIE1-infected HFFs at 48 hpi (Fig. 4b and Supplementary Data 4 and 5). Consistent with previous results, we detected a significant overall protein citrullination in AD169-infected cells at 48 hpi, with IFIT1, IFIT2, and IFIT3, and Mx1 being among the most highly deiminated cellular proteins compared to uninfected control cells (Fig. 4b, left panel). In contrast, upon infection with AD169ΔIE1, we observed a 6.3-, 3.5-, and 2.2-fold decrease in IFIT1, Mx1, and IFIT3 enrichment, respectively (Fig. 4b, right panel), which further supports the crucial role played by the IE1 protein in PAD-mediated citrullination during HCMV infection.

Fig. 2 Cl-A blocks HCMV replication. a HFFs were infected with HCMV (MOI 0.1 PFU/cell) and then treated with increasing concentrations of Cl-A, 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 a percentage relative to untreated controls. The number of plaques is plotted as a function of Cl-A 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-A concentration using the MTT method (black line). Values are expressed as means ± SEM (error bars) of three independent experiments (untreated vs. 25 μM Cl-A $P < 0.001$, untreated vs. 50 μM Cl-A $P < 0.001$, untreated vs. 75 μM Cl-A $P < 0.001$, untreated vs. 100 μM Cl-A $P < 0.001$, untreated vs. 200 μM Cl-A $P < 0.001$, one-way ANOVA followed by Bonferroni's post test). **b** To determine the number of viral DNA genomes in HCMV-infected HFFs, viral DNA was isolated at 144 hpi and analyzed by qPCR using primers amplifying a segment of the IE1 gene. GAPDH was used to normalize HCMV genome counts. Values are expressed as mean ± SEM of three independent experiments (vehicle vs. 25 μM Cl-A $P < 0.001$, vehicle vs. 50 μM Cl-A $P < 0.001$, vehicle vs. 75 μM Cl-A $P < 0.001$, vehicle vs. 100 μM Cl-A $P < 0.001$, vehicle vs. 200 μM Cl-A $P < 0.001$, 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-A or vehicle. Viral supernatants were collected at 144 hpi and analyzed by standard plaque assay. Values are expressed as mean ± SEM of three independent experiments (MOI 1: untreated vs. vehicle $P < 0.001$, untreated vs. Cl-A $P < 0.001$, two-way ANOVA followed by Bonferroni's post test). **d** Protein lysates from uninfected (mock) or infected HFFs (48 and 96 hpi) with (+) or without (−) 100 μM Cl-A were subjected to immunoblotting using the anti-peptidylcitrulline F95 antibody to detect citrullinated proteins, anti-pp28 to assess HCMV infection, or anti-α-tubulin (α-TUB) 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-A. 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 ± SEM (error bars) of three independent experiments (48 hpi: untreated vs. Cl-amidine $P < 0.001$, 72 hpi: untreated vs. Cl-A $P < 0.001$, 72 hpi: untreated *vs.* vehicle $P < 0.05$, 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-A (+) or vehicle (−) were analyzed by immunoblotting for viral expression (IE1-72 kDa, UL44, and pp28) and normalized to α-tubulin (α-TUB). One representative blot of three independent experiments is shown. Data are shown as the mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

The IFN-inducible proteins IFIT1 and Mx1 exert antiviral activity against 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[28]. Specifically, Li and Swaminathan[29] 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. demonstrated that IFIT1 overexpression significantly impairs HCMV replication in astrocytes, whereas IFIT1 knockdown sustains the viral cycle of HCMV[30].

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[31]. Interestingly, MX2 has been recently shown to display antiviral activity against herpesviruses[32,33].

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 or IFIT1—the latter having been shown to exert antiviral activity against HCMV only in fetal astrocytes upon lentiviral overexpression[30]. Following transfection with specific siRNAs against IFIT1 or Mx1 (siIFIT1, siMx1) and subsequent infection with HCMV for 48 h, we achieved a ~60% reduction in IFIT1 protein expression and near to complete silencing of Mx1 protein (Supplementary Fig. 6a). Importantly, in both cases, gene silencing resulted in 1-log higher levels of virus production compared to siCTRL-transfected cells (Fig. 5a).

Given the emerging evidence supporting an antiviral role of IFIT1 against HCMV infection, we asked whether ectopic expression of IFIT1 would curb viral spread in our system. To test this hypothesis, HFFs were transduced with AdVIFIT1 or LacZ as a control at an MOI of 10 and, 24 h post transduction (hpt), infected with HCMV at an MOI of 1 for 144 h. As expected, overexpression of IFIT1 (Supplementary Fig. 6b) reduced viral titer by >2-log compared to AdVLacZ control (Fig. 5b), indicating that IFIT1 strongly impairs HCMV infection in HFFs.

In vitro citrullination of IFIT1 inhibits its binding to triphosphorylated RNA. In order to elucidate the effect of citrullination on IFIT1, we exploited its well-established ability to preferentially bind viral RNA harboring a triphosphate group at its C-terminus (5'-ppp-RNA)[34], which has been shown to be detrimental for the growth of some RNA viruses containing 5'-ppp-RNA. Interestingly, the arginine at position 187 in a highly charged carboxy-terminal groove appears necessary for IFIT1 binding[35]. In this context, we first attempted to determine whether this arginine 187 could be citrullinated by PAD2 after assessment of its in vitro citrullination using the Rh–PG (Supplementary Fig. 6c). LC-MS/MS peptide analysis of recombinant IFIT1 mock or treated with PAD2 for 3 h identified several arginine positions citrullinated in vitro by PAD2, including arginine 187 (Supplementary Data 6). We then conducted a gel shift assay to assess the ability of IFIT1 to bind to 5'-ppp-RNA according to its citrullination state. In vitro transcribed 5'-ppp-RNA was incubated with recombinant IFIT1, either mock or treated with PAD2, and then loaded on an agarose gel. As expected, mock IFIT1 induced a delay in 5'-ppp-RNA migration in comparison with control RNA, indicating the formation of an IFIT1-RNA complex. This effect was reduced when IFIT1 was previously treated with PAD2 for 3 h (Fig. 5c).

In vitro citrullination of UL44 affects long-chain DNA synthesis. To determine whether citrullination could also affect viral protein activity, we measured the ability of native vs. citrullinated forms of VPAP, also known as ICP36 or UL44, to cooperate with the viral DNA polymerase UL54 in promoting long-chain DNA synthesis[36,37], using poly (dA)-oligo $(dT)_{12-18}$ as primer template (Fig. 5d, and Supplementary Fig. 6d). The choice of VPAP was due to its high deimination rate observed at both 48 and 96 hpi compared to mock control (Fig. 4a, and Supplementary Data 1 and 3). To perform this assay, we employed a truncated form of UL44 (UL44ΔC290) retaining all known biochemical activities of full-length UL44 for DNA polymerase stimulation of full-length UL44[38]. As expected, in the presence of UL54 alone we could not detect long-chain DNA, whereas long-chain DNA synthesis was readily detected when both UL54 and UL44ΔC290 were added to the reaction (Fig. 5d and Supplementary 6d). Notably, PAD2- or PAD4-mediated citrullination of UL44ΔC290 reduced the

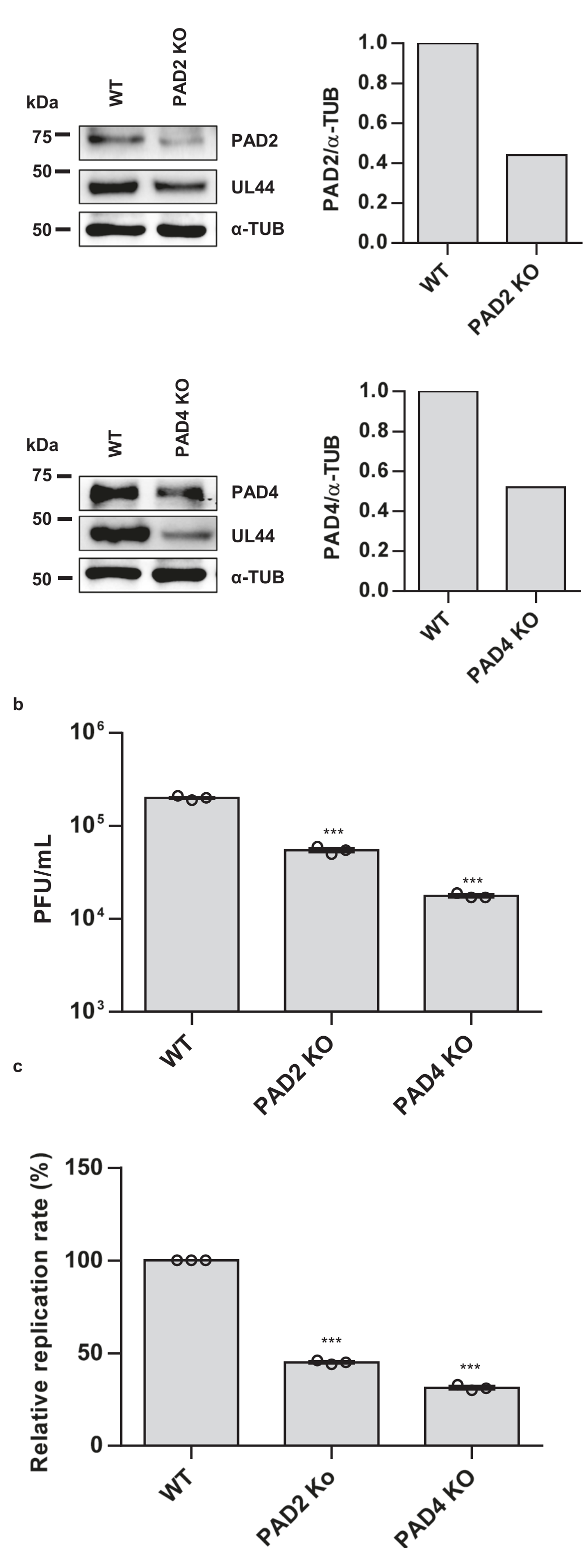


Fig. 3 Effect of PAD2 and PAD4 gene knockout on HCMV replication. a Knockout (KO) gene variants in HFFs for PAD2 (PAD2 KO) and PAD4 (PAD4 KO) were generated using CRISPR/Cas9 technology. The efficiency of PAD2 and PAD4 protein depletion at 48 hpi was assessed by immunoblotting using antibodies against PAD2, PAD4, or α-tubulin (α-TUB), for equal loading. An anti-UL44 antibody was used to verify HCMV infection. The western blot and relative densitometric analysis are representative of three independent experiments. Values are expressed as fold change in PAD2 and 4 expression normalized to α-tubulin (α-TUB). **b** 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 ± SEM of three independent experiments (WT vs. PAD2 KO $P < 0.001$, WT vs. PAD4 KO $P < 0.001$, one-way ANOVA followed by Bonferroni's post test). **c** 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 qPCR with primers amplifying a segment of the IE1 gene. GAPDH was used to normalize HCMV genome counts. Values are expressed as mean ± SEM of three independent experiments (WT vs. PAD2 KO $P < 0.001$, WT vs. PAD4 KO $P < 0.001$, one-way ANOVA followed by Bonferroni's post test). Data are shown as the mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

synthesis of long-chain DNA substantially (Fig. 5d and Supplementary Fig. 6d). This inhibition was not due to carryover of PAD2 or PAD4 because addition of PAD2 and 4 to the reaction did not inhibit DNA synthesis (Fig. 5d and Supplementary 6d). Finally, in vitro citrullination of UL44ΔC290 was confirmed by reduced mobility on SDS-PAGE compared to untreated or mock-treated controls (Supplementary Fig. 6e), as shown previously for other citrullinated proteins[39].

Discussion

Citrullination is an irreversible PTM catalyzed by PADs enzymes. This modification results in the conversion of an arginine side chain into citrulline, with loss of one positive charge and consequent reduction in protein net charge. This PTM can in turn increase protein hydrophobicity and alter intra- and intermolecular interactions, affecting protein conformation, stability, and binding of a wide range of proteins involved in numerous physiological processes, such as apoptosis, differentiation, and gene regulation[10]. Eventually, these structural changes can lead to the gain or loss of protein functions[40]. In this study, we have uncovered the role of citrullination in promoting viral persistence through the deimination of cellular proteins endowed with antiviral activity. Among these, we have focused our attention on IFIT1 and Mx1, two IFN-stimulated genes (ISGs) with a well-established inhibitory activity against various DNA and RNA viruses[28–33]. We show that silencing of either gene results in augmented virus production, whereas ectopic expression of IFIT1 inhibits viral growth, in good agreement with previous results obtained using KSHV-infected cells[29]. Finally, we report that PAD-mediated in vitro deimination of IFIT1 severely affects its ability to bind to 5'-triphosphorylated RNA, thereby impairing the antiviral activity of this protein.

Taken together, our findings suggest that HCMV can trigger PAD-mediated deimination of arginine residues of cellular proteins in order to enhance viral growth, a possibility supported by previous work showing that the arginine at position 187 of the IFIT1 protein is necessary for its binding to 5'-triphosphorylated RNA[34]. This residue is located within a highly charged carboxy-terminal groove that allows recognition and sequestration of viral nucleic acids. Thus, in future studies it will be crucial to characterize the yet unknown mechanisms of IFIT1- and Mx1-mediated inhibition of HCMV growth and to ascertain whether citrullination can hijack these defense pathways in vivo.

Noteworthy, we demonstrate that HCMV IE1 protein is a pivotal factor for PAD induction and subsequent protein citrullination. In this regard, our citrullinome analysis reveals that a variety of cellular proteins, such as IFITs or Mx1, are significantly less citrullinated upon infection with the AD169ΔIE1 mutant compared to the wild-type virus, implying a central role of IE1 in mediating HCMV-induced citrullination events. The residual amount of citrullinated—IFN-inducible—proteins still detectable

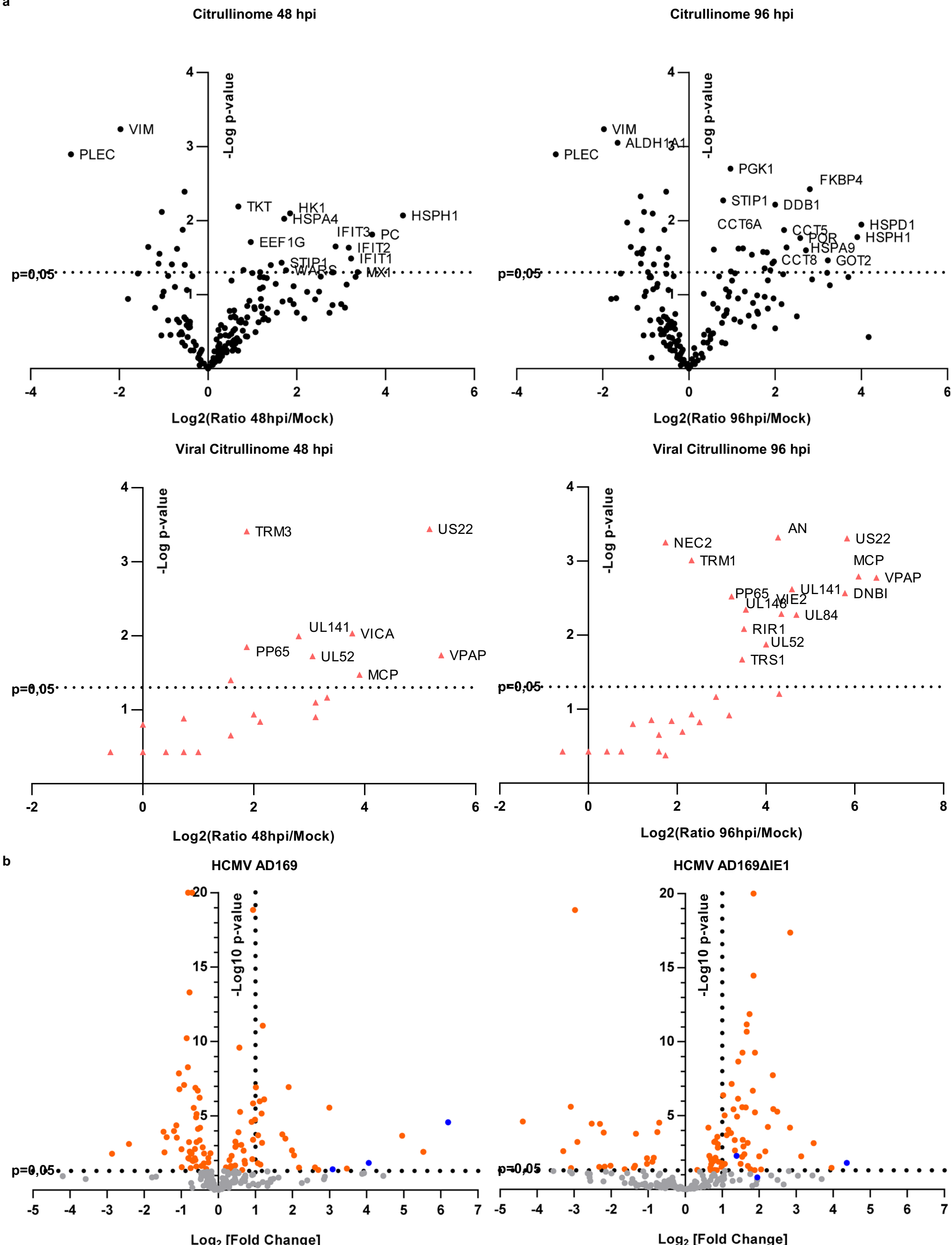


Fig. 4 Characterization of the citrullinated proteome (citrullinome) of HCMV-infected cells. a Volcano plot depicting the host (upper panel—circle) and viral (lower panel—triangle) citrullinated proteins of infected cells vs. mock-infected cells at 48 hpi (left panel) and 96 hpi (right panel). Cell lysates 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/MS—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** Volcano plot depicting the host citrullinated proteins in HCMV AD169- (left panel) and AD169ΔIE1- (right panel) vs. mock-infected HFFs at 48 hpi. Cell lysates from uninfected (mock) or HCMV-infected (MOI 1) HFFs 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/MS—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 ($n = 3$).

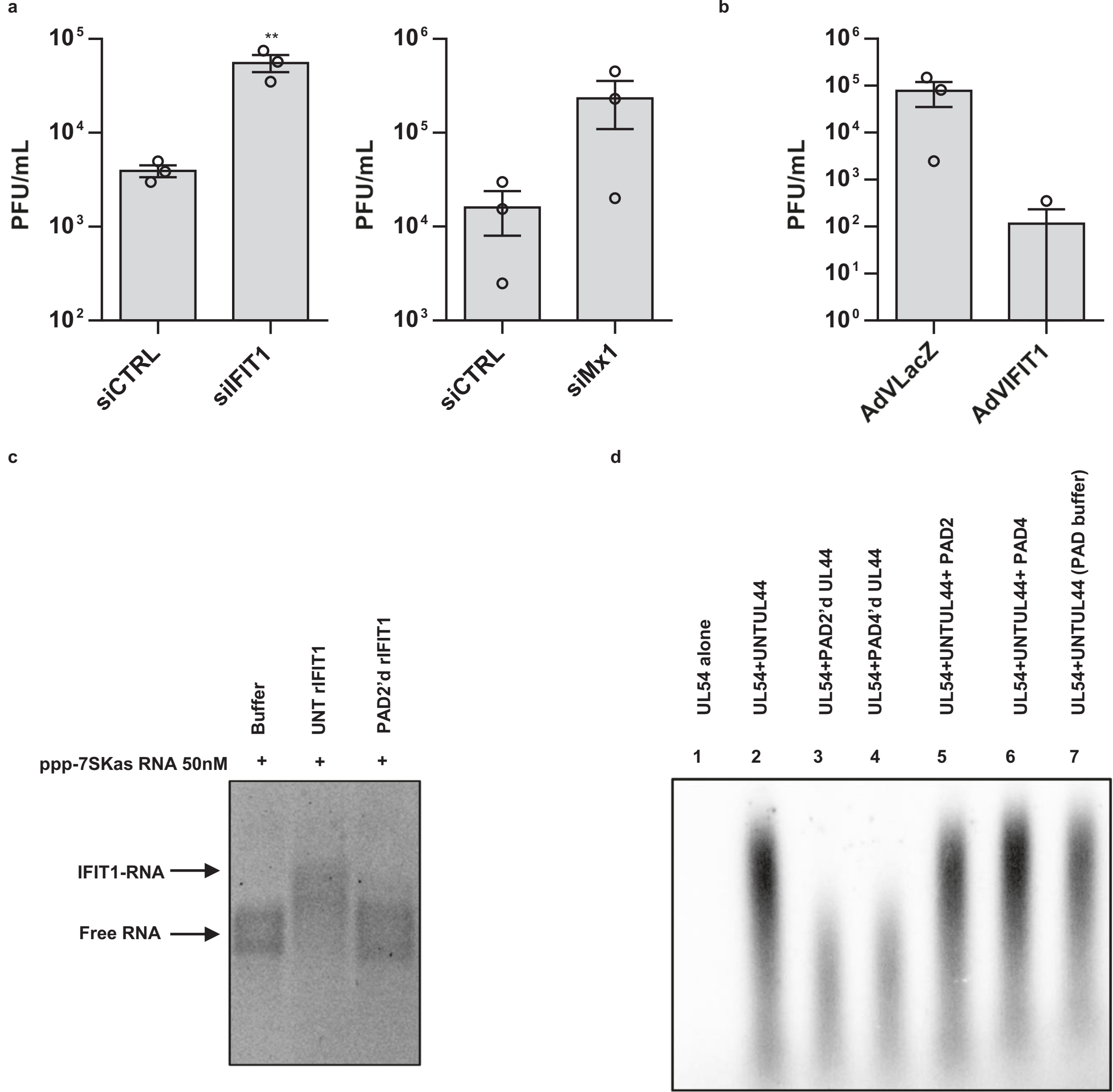


Fig. 5 The antiviral role of IFIT1 and Mx1 against HCMV. a HFFs were silenced for IFIT1 and Mx1 using specific siRNAs (siIFIT1, siMx1, respectively). As negative control cells were also similarly transfected with scrambled siRNA (siCTRL). At 24 hpt, cells were infected with HCMV at an MOI of 0.1 PFU/cell. Viral supernatants were collected at 144 hpi and analyzed by standard plaque assay. Values are expressed as means ± SEM of three independent experiments (siCTRL vs. siIFIT1 $P = 0.0111$, unpaired two-tailed *t* test; siCTRL vs. siMx1 $P > 0.05$, unpaired two-tailed *t* test). **b** HFFs were transduced with AdVIFIT1 or AdVLacZ at an MOI of 10 PFU/cell. Subsequently, cells were infected with HCMV at an MOI of 1. The extent of virus replication was measured at 144 hpi. Results are expressed as means ± SEM of three independent experiments (AdVLacZ vs. AdVIFIT1 $P > 0.05$, unpaired two-tailed *t* test). **c** Gel electrophoretic mobility shift assay of IFIT1 binding to PPP-7SKas RNA. 50 nM of in vitro transcribed 7SK 5′-ppp-RNA was incubated with only buffer (buffer), 5 μM of recombinant IFIT1, either untreated (UNT rIFIT1) or treated with PAD2 (PAD2′d rIFIT1) and loaded on a tris-glycine agarose gel. Data are representative of three experiments. **d** Long-chain DNA synthesis directed by UL54 in the presence or absence of purified UL44ΔC290 was assayed by measuring the incorporation of labeled [$_{32}$P]TTP with a poly(dA)-oligo(dT) primer template. DNA products were resolved on an alkaline agarose gel that was exposed to film and, for quantification, to a phosphorescence screen followed by scanning with a Typhoon scanner. The image shows products directed by UL54 alone, lane 1), or by UL54 in the presence of untreated (UNT) UL44ΔC290 (lane 2), in the presence of UL44ΔC290 treated with PAD2 or PAD4 (PAD2′d, PAD4′d; lanes 3 and 4, respectively), in the presence of UNT UL44ΔC290 but with 270 pM PAD2 (lane 5) or 370 pM PAD4 (lane 6) added to the DNA synthesis reaction, or in the presence of UL44 incubated in PAD reaction buffer, but without PADs (mock-PAD, lane 7). One representative gel of three independent experiments is shown. Data are shown as the mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

upon infection with AD169ΔIE1 suggests that other (viral) proteins may be implicated in PAD induction or that a small number of inactive PADs may be present inside the cell and still capable of citrullinating certain targets upon HCMV-mediated activation. Our findings are consistent with a recent study by Casanova et al. showing that HRV infection upregulates PAD activity and that citrullination of the human cathelicidin LL-37 suppresses its antiviral activity against HRV infection, corroborating the role of citrullination as a viral evasion mechanism employed by certain viruses. Noteworthy, citrullination of this host defense peptide has been shown to disrupt its antimicrobial role, both against bacterial and viral infections, including HRV, and increase its susceptibility to degradation[41–46].

Another important observation of our study is that, upon cellular entry, several viral proteins are citrullinated as well, raising the hypothesis that citrullination may concomitantly

influence the activity of both viral and cellular proteins. Among viral proteins, we find that in vitro citrullination of UL44, an accessory protein that increases the DNA processivity of UL54[36,37], reduces its ability to drive long-chain DNA synthesis. Although the physiological role of UL44 citrullination during HCMV infection cannot be inferred from the results of this study, it is tempting to speculate that HCMV may exploit UL44 citrullination to fine-tune its DNA replication during specific phases of viral infection.

Overall, our observation that HCMV growth relies, in part, on PAD-mediated citrullination suggests the attractive prospect that deimination of host—and possibly—viral proteins may be exploited for the design of antiviral agents against HCMV and, more in general, herpesviruses. An alternative pharmacological strategy—perhaps less costly and time-consuming—could rely on repurposing existing PAD inhibitors, such as Cl-A, which we have shown to have a potent inhibitory effect against HCMV-induced citrullination and viral growth.

As HCMV has been implicated in various autoimmune, inflammatory, and cardiovascular diseases as well as cancer development, all characterized by a high degree of protein citrullination, it is possible that HCMV-mediated protein citrullination also constitutes a key event in the pathogenesis of such diseases.

Methods

Cells and viruses. Human foreskin fibroblasts (HFFs, ATCC SCRC-1041™), African green monkey kidney cells (Vero; Sigma-Aldrich 84113001), human HEK 293 from the human kidney (embryonic) (HEK 293, Sigma-Aldrich 85120602), HEK 293T (HEK 293T, ATCC® CRL-3216™) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich, Milan, Italy). CD4 + lymphoblastoid T (C8166, ECACC 88051601) were kept in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% FCS (Sigma-Aldrich, Milan, Italy).

HCMV strain Merlin, kindly provided by Gerhard Jahn and Klaus Hamprecht (University Hospital of Tübingen, Germany), and the HCMV laboratory strain AD169 (ATCC-VR538), were propagated and titrated on HFFs[47]. AD169ΔIE1 was kindly provided by Thomas Stamminger (University of Ulm, Germany). UV-inactivated Merlin was prepared using a double pulse of UV-B light (1.2 J/cm^2).

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[48]. HIV-1_{IIIb} strain stock was prepared in C8166 cells, as previously described[49].

Recombinant adenoviral vectors (AdV) encoding HCMV IE2 (AdVIE2) and *Escherichia coli* β-galactosidase (AdVLacZ) have been previously described[47,50], while AdV-IE72 (AdVIE1) was provided by Dr. Timothy F. Kowalik (University of Massachusetts Medical School, Worcester, MA)[51]. Recombinant AdV stocks were propagated and titrated in HEK 293 by standard plaque assay[47,50,51].

Reagents and proteins. Recombinant human PAD2, PAD4, and Cl-A were from Cayman Chemical (Ann Arbor, USA). Cycloheximide and Foscarnet were from Sigma-Aldrich (Milan, Italy). BB-Cl-A was kindly provided by P. R. Thompson (University of Massachusetts, Medical School).

The plasmid pET30A hIFIT1-GST-His was kindly provided by A. Pichlmair (Technische Universitaet Munich, Germany). pSGIE72 IE1-encoding plasmid was used in the quantitative nucleic acid analysis. The pSicoR-CRISPR-Cas9 vector (RP-557) was kindly provided by R. J. Lebbink (UMC, Utrecht).

In vitro antiviral assay. HFFs, HEK 293, and Vero were incubated with increasing concentrations of Cl-A (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-A 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 by standard plaque assay on HFFs for HCMV, on HEK 293 for adenovirus, and on Vero cells for HSV-1/HSV-2[47,48].

Cell viability assay. To determine cell viability, HFFs, HEK 293, and Vero were exposed to increasing concentrations of Cl-A. 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[52]. Briefly, MTT solutions from the Stock (final concentration 500 μg/ml) were added, and cells were incubated in a CO_2 incubator in the dark for 2 h. Then, the medium was removed and the resulting formazan crystals were dissolved using 100 μl of DMSO. Finally, the resulting colored solution was transferred in a 96-well plate, and the absorbance was read at 570 nm using 630 nm as reference wavelength on a Victor X3 Multi-label Plate Reader (Perkin Elmer, Waltham, MA, USA; wallac 1420 work station software). The viability of the C8166 cells in presence of scalar concentrations of Cl-A was determined by the 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 the increasing amount of Cl-A (0, 10, 25, 75, 100, or 200 μM) and then added to C8166 cells (0.5×10^6 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 a fresh medium plus the same drug concentration used in the pre-incubation. The HIV-1 gag p24 amount was determined at day 7 pi in the culture supernatants with the HIV-1 p24 antigen ELISA kit (Biomerieux, Marcy-l'Étoile, France). Mock-infected C8166 cells were used as a 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 Mx3000P 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[53]. Briefly, equal amounts of cell extracts were fractionated by electrophoresis on SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Merck Millipore, Burlington, MA, USA). After blocking with TBS (Tris-buffered saline containing 0.05% Tween20) containing 5% milk, membranes were incubated overnight at 4 °C with the appropriate primary antibodies. In this study, the primary antibodies used were: anti-peptidyl-citrulline, clone F95 (1:500), anti-IFIT1 (1:500), anti-V5 (1:1000), anti-pp65 (1:1000), anti-pp28 (1:1000), anti-UL44 (1:1000), anti-Mx1 (1:500), anti-IEA (1:1000), anti-PAD2 (1:1500), anti-PAD6 (1:500), anti-PAD4 (1:500), anti-PAD3 (1:500), anti-PAD1 (1:500), anti-actin clone C4 (1:1000), and anti-α-tubulin (1:1000). The primary antibodies used are detailed in Supplementary Table 2. Membranes were then washed and incubated for 1 h at room temperature with secondary antibodies: anti-rabbit/mouse IgG and anti-mouse IgM, horseradish peroxidase-linked species-specific whole antibody (Amersham, Merck Life Science S.r.l., Milan, Italy) or goat anti-mouse IgM antibody (Merck Life Science S.r.l., Milan, Italy) (1:2000). Proteins were visualized using ChemiDoc MP Imaging System (Bio-Rad Laboratories Srl), and an enhanced chemiluminescence detection kit (Thermo Fisher Scientific, Waltham, MA, Stati USA). Scanning densitometry of the bands was performed using Image Lab (version 4.6.9; Bio-Rad Laboratories S.r.l., Segrate, Italy).

Detection of citrullination with rhodamine–phenylglyoxal (Rh–PG). Equal amounts of protein were diluted with 80% trichloroacetic acid and incubated with Rh–PG (final concentration 0.1 mM) for 30 min[25]. The reaction was quenched with 100 mM L-citrulline, then centrifuged at 21,100×*g* for 10 min and washed with ice-cold acetone, and resuspended in 2× SDS loading dye for gel electrophoresis. Gels were imaged (excitation = 532 nm, emission = 580 nm) using a ChemiDoc MP Imaging System (Bio-Rad Laboratories Srl), 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 1× TBST (Tris-buffered saline, 0.05 % Tween20). Then, 50 μg of protein lysates from mock- or HCMV-infected HFFs at different time points, in the presence or absence of 100 μM Cl-A or with the same volume of the vehicle as a negative control, were diluted in calcium-free PAD reaction buffer (2 mM DTT, 50 mM NaCl,100 mM Tris, pH 7.57). As a 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 1× 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, UK) and anti-rabbit IgG-Fc HRP-conjugated secondary antibody were used to detect citrullinated proteins (1:7000). 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 of incubation, which changed to yellow once stopped by the addition of 1 N HCl. The optical density (OD) was measured at 450–620 nm using a Victor X3 Multilabel Reader (Perkin Elmer, Waltham, MA, USA; wallac 1420 work station software)[54,55].

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 3. The thermocycler (Biorad C1000 Touch Thermal Cycler) settings consisted of 30 s of 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 s, 56 °C for 30 s, and 72 °C for 4 min) and a final extension at 72 °C for 8 min. The resulting amplification products were digested with XhoI (Thermo Fisher Scientific, Waltham, USA) and HindIII (Thermo Fisher Scientific, Waltham, USA) and cloned into the pGL4.20[luc2/Puro] Vector (Promega, Madison, USA), 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. The resulting chromatograms were analyzed using Chromas software 2.6.6 (Technolysium Ltd.).

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 (Promega, Madison, USA), 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 Victor X3 Multilabel Plate Reader (Perkin Elmer, Waltham, MA, USA; wallac 1420 work station software). 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 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 guide RNA (gRNA) consisting of a gene-specific CRISPR RNA (crRNA) fused to the trans-activating crRNA (tracrRNA) and a terminator sequence[56]. gRNA sequences are reported in Supplementary Table 4. An empty vector carrying no gRNA was used as negative control (WT cell line). All constructs were verified by Sanger sequencing (Chromas 2.6.6).

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, Italy) 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 μg/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. In addition, indel frequencies were quantified using TIDE[57]. 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. 3). 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 of genomic DNA and Q5 High-Fidelity DNA polymerase according to the manufacturer's 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) (Biorad C1000 thermocycler). 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 5.

Pull down. Uninfected or HCMV-infected cells (MOI of 1 PFU/ml) were washed with 1× 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.

Citrullinome analysis by mass spectrometry: sample preparation. Sample preparation in technical triplicates followed the procedure outlined in ref. [58]. 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[59] for 30 min at 37 °C. Labeled proteomes were precipitated on ice for 30 min. Samples were pelleted through tabletop centrifugation (21,100×*g*, 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 1× PBS to a 0.2% SDS final concentration. 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 (5 mL) in order to remove any unbound proteins. Beads were then transferred to a screw-cap microcentrifuge tube and heated in 1× 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 (1400 ×*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, Madison, USA) 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 supernatant. 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 (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an Easy-nLC HPLC (Thermo Fisher Scientific, Waltham, MA, USA). 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 seven 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 and viral 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.

Reductive dimethylation (ReDiMe) labeling and mass spectrometry for the citrullinome analysis of wtAD169 and AD169ΔIE1-infected cells. The trypsin digest was desalted using Pierce C18 spin column (catalog No 89870) according to the manufacturer's protocol and was resuspended in 200 μL of 100 mM triethylammonium bicarbonate, pH 8.5. 20% $H^{12}CHO$ (4 μL, light formaldehyde), $D^{12}CDO$ (4 μL, medium formaldehyde), and $D^{13}CDO$ (4 μL, heavy formaldehyde) were added to the mock, AD169- and AD169ΔIE1-infected samples, respectively. In total, 20 μL of 0.6 M sodium cyanoborohydride ($NaBH_3CN$) was then added to both the mock and AD169-infected samples, respectively, while 20 μL of 0.6 M sodium cyanoborodeuteride ($NaBD_3CN$) was added to the AD169ΔIE1-infected samples. The samples were incubated at room temperature for 2 h. The samples were then cooled on ice and the reaction quenched with 4 μL of 20% ammonium hydroxide. Formic acid (8 μL) was then added to the samples. Heavy, medium, and light formaldehyde-labeled samples were mixed together, and the mixture was desalted and stored at −20 °C for proteomic analysis. Data was acquired using a NanoAcquity UPLC (Waters Corporation, Milford, MA) coupled to an Orbitrap Fusion Lumos Tribrid (Thermo Fisher Scientific, Waltham, MA) mass spectrometer. Peptides were trapped and separated using an in-house 100 μm I.D. fused-silica pre-column (Kasil frit) packed with 2 cm ProntoSil (Bischoff

Chromatography, DE) C18 AQ (200 Å, 5 μm) media and configured to an in-house packed 75 μm I.D. fused-silica analytical column (gravity-pulled tip) packed with 25 cm Magic (Bruker, Billerica, MA) C18-AQ (100 Å, 3 μm) media, respectively. Mobile phase A was water supplemented with 0.1 % (v/v) formic acid, and mobile phase B was acetonitrile supplemented with 0.1 % (v/v) formic acid. Following a 3.8 μL of sample injection, peptides were trapped at flow rate of 4 μL/min with 5% B for 4 min, followed by gradient elution at a flow rate of 300 nL/min from 5 to 35% B over 120 min (total run time 145 min). Electrospray voltage was delivered by a liquid junction electrode (1.5 kV) located between the columns and the transfer capillary to the mass spectrometer was maintained at 275 °C. Mass spectra were acquired over *m/z* 375–1500 Da with a resolution of 120,000 (*m/z* 200), a maximum injection time of 110 ms, and an AGC target of 400,000. Tandem mass spectra were acquired using data-dependent acquisition (3 s cycle) with an isolation width of 1.6 Da, HCD collision energy of 30%, resolution of 15,000 (*m/z* 200), maximum injection time of 50 ms, and an AGC target of 50,000.

Database search. Raw data were processed and searched using Maxquant 1.6.14 and its integrated Andromeda search engine using the Swiss-Prot human (downloaded 04/09/2019) and Uniprot HCMV (downloaded 02/27/2021). Search parameters were as follows: tryptic digestion with up to 2 missed cleavages; peptide N-terminal acetylation, methionine oxidation, N-terminal glutamine to pyroglutamate conversion were specified as variable modifications. The monoisotopic mass increment of the triplex dimethyl labels, light, medium, and heavy dimethyl labels at 28.0313, 32.0564, and 36.0757 Da, respectively, were set as a variable modification on the peptide N-termini and lysine residues. Carbamidomethylation of cysteines was set as static modification. The main search tolerance was 6 ppm, and the first search tolerance was 50 ppm. Both the protein and peptides identification false discovery rates (FDR) were <1%. Protein grouping, dimethyl ratio calculations, and downstream statistics were performed in Scaffold Q + S 4.8.9 (Proteome Software, Portland, OR).

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. Nos.: SI02781093, SI05459538, SI04435963, SI04435956), PAD2 (siPAD2, FlexiTube siRNAs cat. Nos.: SI04278953, SI04255860, SI04188793, SI00676872), PAD4 (siPAD4, FlexiTube siRNAs cat. Nos.: SI04939032, SI04359530, SI04299288, SI00676907) or control siRNA (siCTRL, 1027292) as a negative control. For siRNA-mediated knockdown of IFIT1, siRNA approach was performed according to Pichlmair et al. (siIFIT1_1: CTCCTTGGGTCGTTCTA CAAA; siIFIT1_2: TACATGGGAGTTATCCATTGA)[34].

Recombinant adenoviral vector production. The adenovirus-derived vectors (AdVs) expressing IFIT1 and LacZ were generated by means of a replacement strategy using recombineering methods, as described previously[60,61]. The open reading frame (ORF) was amplified using a specific set of primers for each desired construct (Supplementary Table 6), and colonies were analyzed by PCR and sequencing (Supplementary Table 6). IFIT1 and LacZ expression was assessed by western blotting using a V5 antibody. For cell transduction, HFFs were incubated with AdVIFIT1 at an MOI of 10 in DMEM. After 2 h at 37 °C, the virus was washed off, and fresh medium was applied. At 24 hpt, HFFs were infected with HCMV strain Merlin at an MOI of 1 and incubated for 144 h.

UL54 and UL44 production and in vitro citrullination. Glutathione-S-transferase (GST) tagged UL54 was produced as described[62] and was kindly provided by Han Chen. UL44ΔC290 was produced as described[63] with several modifications: after resuspension, cells were lysed by sonication at an amplitude of 40% for a total of ~15 min, pulsing in cycles of 5 s on and 9 s off. The lysate was centrifuged at 18,000×*g* for 1.5 h at 4 °C, then applied to a glutathione-Sepharose 4 FastFlow column (GE Healthcare) that had been equilibrated in buffer A (50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 2 mM dithiothreitol (DTT), 500 mM NaCl, 20% glycerol), and then washed extensively with the same buffer. UL44ΔC290 was eluted with buffer B (Buffer A + 15 mM glutathione), and fractions containing the protein were combined and diluted 1.5:1 with buffer C (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 2 mM DTT, 10% glycerol). To cleave off the GST tag, the sample was treated with HRV 3C protease (Takara) overnight at 4 °C, loaded onto a heparin agarose column (Protein Ark), and eluted with a linear gradient of 150–1000 mM NaCl in buffer C. Fractions containing UL44ΔC290 were combined and applied to a HiLoad® Superdex® 200 column (GE Healthcare) in storage buffer (50 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 2 mM DTT, 500 mM NaCl, 20% Glycerol). Fractions containing UL44ΔC290 were combined, concentrated using an Amicon® Ultra-15 Centrifugal Filter Device (Merck Millipore, Milan), and stored in aliquots at −80 °C.

In vitro citrullination of UL44 was performed as follows: 6 μM UL44ΔC290 was incubated in PAD assay buffer (50 mM HEPES, pH 7.6, 10 mM $CaCl_2$, 150 mM NaCl, 2 mM DTT, 12.5% glycerol for UL44) with recombinant human PAD2 or PAD4 (Chemical Cayman) at a concentration of 0.7 μM for 180 min, at 37 °C. Citrullination was terminated by the addition of 0.05 M EDTA. As a control, a reaction was performed without PAD enzymes (mock treatment). Samples were purified on a Superose 6 Increase GL column (GE Healthcare) in 50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 2 mM DTT, 150 mM NaCl, 10% glycerol, to remove the vast majority of the PAD enzymes. Fractions from each reaction containing UL44ΔC290 were concentrated using an Amicon® Ultra-15 Centrifugal Filter Device (Merck Millipore, Milan), and stored at −20 °C. In order to estimate the amount of residual PAD enzymes, 2.2 μg of each UL44ΔC290 sample was applied to SDS-PAGE, stained with Coomassie brilliant blue, and bands corresponding to PAD2 or PAD4 contamination were quantified relative to known amounts of PAD2 and PAD4 (Image Lab).

Production of recombinant human IFIT1 and in vitro citrullination. Recombinant IFIT proteins were expressed in *E. coli* and were purified on a HisTrap HP column (GE Healthcare, Chicago, IL, Stati Uniti).

The in vitro citrullination of IFIT1 was performed as previously described for UL44.

LC-MS/MS analysis of mock- or PAD2-treated recombinant IFIT1. One μg of recombinant IFIT1, mock or PAD2-treated, was digested with 1:50 LysC (Wako #129-02541) overnight at 37 °C. Peptides were purified and concentrated on stage tips with three C18 Empore filter discs (3 M), and further measured via LC-MS/MS, using an EASY- nLC 1200 system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Q-Exactive HF mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), on a 20-cm reverse-phase analytical column (75 μm column diameter; ReproSil-Pur C18-AQ 1.9-μm resin; Dr. Maisch) and separated using a 60 min acetonitrile gradient. Raw files were processed using MaxQuant version 1.6.0.15 with carbamidomethyl (Cys) as fixed modification as well as oxidation (Met), Acetyl (Protein N-term) and citrullination (R), as defined by H(-1) N(-1) O, as variable modifications.

In vitro transcription 7SK 5′-PPP-RNA and gel shift assay. Non-coding RNA (7SK anti-sense 356 bp) was transcribed in vitro using the SP6 MEGAscript kit (Thermo Fisher Scientific, Waltham, MA, USA)) as per the manufacturer's instruction[64]. In all, 50 nM (5 pmol) of in vitro transcribed 7SK 5'-ppp-RNA was incubated with 5 μM of recombinant IFIT1, either mock or treated with PAD2 for 3 h, for 30 min at room temperature in PBS—400 mM DTT—100 mM NaCl, and loaded on a 0.8% Tris-Glycine agarose gel. The gel was stained in a 3× Gel Red (Biotium, #41003) solution before imaging.

Functional assay of UL44. Long-chain DNA synthesis by UL54 and UL44 on a poly(dA)-oligo$(dT)_{12-18}$ primer template (Amersham Biosciences, Little Chalfont, UK) with radiolabeled $[^{32}P]$dTTP (~55 Ci/mmol) as a substrate was measured using a modification of a gel-based assay described previously[38]. Reactions contained 50 mM TRIS-HCl [pH 7.5], 100 mM $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 4 % Glycerol, 40 μg/mL BSA, and using 26.5 nM GST-UL54 alone or with 58 nM of untreated UL44ΔC290 (UNT control), or with PAD2-treated UL44ΔC290, PAD4-treated UL44ΔC290, or mock-treated UL44ΔC290. To control for any effects of trace amounts of PAD enzymes contaminating UL44ΔC290 purified from citrullination reactions, in separate mixtures UNT control reactions were supplemented with 270 pM PAD2 or 370 pM PAD4. Gels with incorporated radioactivity were exposed to phosphorescence screens that were scanned with a Typhoon scanner (Amersham Biosciences, Little Chalfont, UK) and quantified using Image Lab. Data were normalized in each experiment by setting the UNT control as 100%. Statistical analysis was performed by one-way analysis of variance (ANOVA) with Dunnett's correction using GraphPad Prism version 8.4.3 for Windows.

GO protein class. 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[65].

Statistical analysis. All statistical tests and the IC50 were performed using GraphPad Prism version 5.00 or 8.4.3 for Windows (GraphPad Software, San Diego, USA). The data were presented as means ± standard error of mean (SEM). Statistical significance was determined by using an unpaired *t* test (two-tailed), one-way or two-way analysis of variance (ANOVA) with Bonferroni's, or Dunnett's post tests. Differences were considered statistically significant for $P < 0.05$ ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data that support the findings of this study are available within the paper and its supplementary information files. All other relevant data are available from the corresponding authors upon reasonable request. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner

repository with the dataset identifier PXD025803, and PXD025818. Source data are provided with this paper.

Received: 7 October 2019; Accepted: 29 May 2021;
Published online: 23 June 2021

References

1. Griffiths, P., Baraniak, I. & Reeves, M. The pathogenesis of human cytomegalovirus. *J. Pathol.* **235**, 288–297 (2015).
2. Britt, W. J. Congenital human cytomegalovirus infection and the enigma of maternal immunity. *J. Virol.* **91**, e02392–16 (2017).
3. Halenius, A. & Hengel, H. Human cytomegalovirus and autoimmune disease. *Biomed. Res. Int.* **2014**, 472978 (2014).
4. Soderberg-Naucler, C. Does cytomegalovirus play a causative role in the development of various inflammatory diseases and cancer? *J. Intern. Med.* **259**, 219–246 (2006).
5. Du, Y., Zhang, G. & Liu, Z. Human cytomegalovirus infection and coronary heart disease: a systematic review. *Virol. J.* **15**, 31 (2018).
6. Jackson, S. E. et al. CMV immune evasion and manipulation of the immune system with aging. *GeroScience* **39**, 273–291 (2017).
7. Cobbs, C. S. Does valganciclovir have a role in glioblastoma therapy? *Neuro. Oncol.* **16**, 330–331 (2014).
8. Herbein, G. The human cytomegalovirus, from oncomodulation to oncogenesis. *Viruses* **10**, 408 (2018).
9. Baker, P. J. et al. Posttranslational modification as a critical determinant of cytoplasmic innate immune recognition. *Physiol. Rev.* **97**, 1165–1209 (2017).
10. Gudmann, N. S., Hansen, N. U. B., Jensen, A. C. B., Karsdal, M. A. & Siebuhr, A. S. Biological relevance of citrullinations: diagnostic, prognostic and therapeutic options. *Autoimmunity* **48**, 73–79 (2015).
11. Bicker, K. L. & Thompson, P. R. The protein arginine deiminases: structure, function, inhibition, and disease. *Biopolymers* **99**, 155–163 (2013).
12. van Venrooij, W. J., van Beers, J. J. B. C. & Pruijn, G. J. M. Anti-CCP antibodies: the past, the present and the future. *Nat. Rev. Rheumatol.* **7**, 391–398 (2011).
13. Vossenaar, E. R. & van Venrooij, W. J. Citrullinated proteins: sparks that may ignite the fire in rheumatoid arthritis. *Arthritis Res. Ther.* **6**, 107 (2004).
14. Knight, J. S. et al. Peptidylarginine deiminase inhibition disrupts NET formation and protects against kidney, skin and vascular disease in lupus-prone MRL/ *lpr* mice. *Ann. Rheum. Dis.* **74**, 2199–2206 (2015).
15. Acharya, N. K. et al. Neuronal PAD4 expression and protein citrullination: possible role in production of autoantibodies associated with neurodegenerative disease. *J. Autoimmun.* **38**, 369–380 (2012).
16. Yang, L., Tan, D. & Piao, H. Myelin basic protein citrullination in multiple sclerosis: a potential therapeutic target for the pathology. *Neurochem. Res.* **41**, 1845–1856 (2016).
17. Nicholas, A. P. Dual immunofluorescence study of citrullinated proteins in Parkinson diseased substantia nigra. *Neurosci. Lett.* **495**, 26–29 (2011).
18. Yuzhalin, A. E. Citrullination in cancer. *Cancer Res.* **79**, 1274–1284 (2019).
19. Sokolove, J. et al. Brief report: citrullination within the atherosclerotic plaque: a potential target for the anti-citrullinated protein antibody response in rheumatoid arthritis. *Arthritis Rheum.* **65**, 1719–1724 (2013).
20. Casanova, V. et al. Citrullination alters the antiviral and immunomodulatory activities of the human cathelicidin LL-37 during rhinovirus infection. *Front. Immunol.* **11**, 85 (2020).
21. Trier, N. H. et al. Antibodies to a strain-specific citrullinated Epstein-Barr virus peptide diagnoses rheumatoid arthritis. *Sci. Rep.* **8**, 3684 (2018).
22. Pratesi, F. et al. Antibodies to a new viral citrullinated peptide, VCP2: fine specificity and correlation with anti-cyclic citrullinated peptide (CCP) and anti-VCP1 antibodies. *Clin. Exp. Immunol.* **164**, 337–345 (2011).
23. Pratesi, F., Tommasi, C., Anzilotti, C., Chimenti, D. & Migliorini, P. Deiminated Epstein-Barr virus nuclear antigen 1 is a target of anti–citrullinated protein antibodies in rheumatoid arthritis. *Arthritis Rheum.* **54**, 733–741 (2006).
24. Arisan, E. D., Uysal-Onganer, P. & Lange, S. Putative roles for peptidylarginine deiminases in COVID-19. *Int. J. Mol. Sci.* **21**, 1–29 (2020).
25. Bicker, K. L., Subramanian, V., Chumanevich, A. A., Hofseth, L. J. & Thompson, P. R. Seeing citrulline: development of a phenylglyoxal-based probe to visualize protein citrullination. *J. Am. Chem. Soc.* **134**, 17015–17018 (2012).
26. Scherer, M. et al. Characterization of recombinant human cytomegaloviruses encoding IE1 mutants L174P and 1-382 reveals that viral targeting of PML bodies perturbs both intrinsic and innate immune responses. *J. Virol.* **90**, 1190–1205 (2016).
27. Luo, Y., Knuckley, B., Lee, Y.-H., Stallcup, M. R. & Thompson, P. R. A fluoroacetamidine-based inactivator of protein arginine deiminase 4: design, synthesis, and in vitro and in vivo evaluation. *J. Am. Chem. Soc.* **128**, 1092–1093 (2006).
28. Fensterl, V. & Sen, G. C. Interferon-induced ifit proteins: their role in viral pathogenesis. https://doi.org/10.1128/JVI.02744-14 (2015).
29. Li, D. & Swaminathan, S. Human IFIT proteins inhibit lytic replication of KSHV: a new feed-forward loop in the innate immune system. *PLoS Pathog.* **15**, e1007609 (2019).
30. Zhang, L. et al. Antiviral effects of IFIT1 in human cytomegalovirus-infected fetal astrocytes. *J. Med. Virol.* **89**, 672–684 (2017).
31. Haller, O., Staeheli, P., Schwemmle, M. & Kochs, G. Mx GTPases: dynamin-like antiviral machines of innate immunity. *Trends Microbiol.* **23**, 154–163 (2015).
32. Schilling, M. et al. Human MxB protein is a pan-herpesvirus restriction factor. *J. Virol.* **92**, JVI.01056-18 (2018).
33. Crameri, M. et al. MxB is an interferon-induced restriction factor of human herpesviruses. *Nat. Commun.* **9**, 1980 (2018).
34. Pichlmair, A. et al. IFIT1 is an antiviral protein that recognizes 5′-triphosphate RNA. *Nat. Immunol.* **12**, 624–630 (2011).
35. Abbas, Y. M. et al. Structure of human IFIT1 with capped RNA reveals adaptable mRNA binding and mechanisms for sensing N1 and N2 ribose 2'-O methylations. *Proc. Natl Acad. Sci. USA* **114**, E2106–E2115 (2017).
36. Ertl, P. F. & Powell, K. L. Physical and functional interaction of human cytomegalovirus DNA polymerase and its accessory protein (ICP36) expressed in insect cells. *J. Virol.* **66**, 4126–4133 (1992).
37. Weiland, K. L., Oien, N. L., Homa, F. & Wathen, M. W. Functional analysis of human cytomegalovirus polymerase accessory protein. *Virus Res.* **34**, 191–206 (1994).
38. Loregian, A., Appleton, B. A., Hogle, J. M. & Coen, D. M. Residues of human cytomegalovirus DNA polymerase catalytic subunit UL54 that are necessary and sufficient for interaction with the accessory protein UL44. *J. Virol.* **78**, 158–167 (2004).
39. Shelef, M. A., Bennin, D. A., Mosher, D. F. & Huttenlocher, A. Citrullination of fibronectin modulates synovial fibroblast behavior. *Arthritis Res. Ther.* **14**, R240 (2012).
40. Tarcsa, E. et al. Protein unfolding by peptidylarginine deiminase: Substrate specificity and structural relationships of the natural substrates trichohyalin and filaggrin. *J. Biol. Chem.* **271**, 30709–30716 (1996).
41. Barlow, P. G., Findlay, E. G., Currie, S. M. & Davidson, D. J. Antiviral potential of cathelicidins. *Future Microbiol.* **9**, 55–73 (2014).
42. Barlow, P. G. et al. Antiviral activity and increased host defense against influenza infection elicited by the human cathelicidin LL-37. *PLoS ONE* **6**, e25333 (2011).
43. Currie, S. M. et al. The human cathelicidin LL-37 has antiviral activity against respiratory syncytial virus. *PLoS ONE* **8**, 73659 (2013).
44. Wang, G., Mishra, B., Epand, R. F. & Epand, R. M. High-quality 3D structures shine light on antibacterial, anti-biofilm and antiviral activities of human cathelicidin LL-37 and its fragments. *Biochimica et. Biophysica Acta—Biomembranes* **1838**, 2160–2172 (2014).
45. Martineau, A. R. et al. IFN-γ- and TNF-independent vitamin D-inducible human suppression of mycobacteria: the role of cathelicidin LL-37. *J. Immunol.* **178**, 7190–7198 (2007).
46. Kilsgård, O. et al. Peptidylarginine deiminases present in the airways during tobacco smoking and inflammation can citrullinate the host defense peptide LL-37, resulting in altered activities. *Am. J. Respir. Cell Mol. Biol.* **46**, 240–248 (2012).
47. Gariano, G. R. et al. The intracellular DNA sensor IFI16 gene acts as restriction factor for human cytomegalovirus replication. *PLoS Pathog.* **8**, e1002498 (2012).
48. Luganini, A., Caposio, P., Landolfo, S. & Gribaudo, G. Phosphorothioate-modified oligodeoxynucleotides inhibit human cytomegalovirus replication by blocking virus entry. *Antimicrob. Agents Chemother.* **52**, 1111–1120 (2008).
49. Bon, I. et al. Peptide-derivatized SB105-A10 dendrimer inhibits the infectivity of R5 and X4 HIV-1 strains in primary PBMCs and cervicovaginal histocultures. *PLoS ONE* **8**, e76482 (2013).
50. Pignoloni, B. et al. Distinct roles for human cytomegalovirus immediate early proteins IE1 and IE2 in the transcriptional regulation of MICA and PVR/CD155 expression. *J. Immunol.* **197**, 4066–4078 (2016).
51. E, X. et al. An E2F1-Mediated DNA damage response contributes to the replication of human cytomegalovirus. *PLoS Pathog.* **7**, e1001342 (2011).
52. Pauwels, R. et al. Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. *J. Virol. Methods* **20**, 309–321 (1988).
53. Dell'Oste, V. et al. Innate nuclear sensor IFI16 translocates into the cytoplasm during the early stage of in vitro human cytomegalovirus infection and is entrapped in the egressing virions during the late stage. *J. Virol.* **88**, 6970–6982 (2014).
54. Zendman, A. J. W. et al. ABAP: antibody-based assay for peptidylarginine deiminase activity. *Anal. Biochem.* **369**, 232–240 (2007).
55. Damgaard, D., Senolt, L., Nielsen, M. F., Pruijn, G. J. & Nielsen, C. H. Demonstration of extracellular peptidylarginine deiminase (PAD) activity in

synovial fluid of patients with rheumatoid arthritis using a novel assay for citrullination of fibrinogen. *Arthritis Res. Ther.* **16**, 498 (2014).
56. van de Weijer, M. L. et al. A high-coverage shRNA screen identifies TMEM129 as an E3 ligase involved in ER-associated protein degradation. *Nat. Commun.* **5**, 3832 (2014).
57. Brinkman, E. K., Chen, T., Amendola, M. & van Steensel, B. Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Res.* **42**, e168 (2014).
58. Tilvawala, R. et al. The rheumatoid arthritis-associated citrullinome. *Cell Chem. Biol.* **25**, 691–704.e6 (2018).
59. Lewallen, D. M. et al. Chemical proteomic platform to identify citrullinated proteins. *ACS Chem. Biol.* **10**, 2520 (2015).
60. Biolatti, M. et al. Human cytomegalovirus tegument protein pp65 (pUL83) dampens type I interferon production by inactivating the DNA sensor cGAS without affecting STING. *J. Virol.* **92**, e01774-17 (2018).
61. Chartier, C. et al. Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. *J. Virol.* **70**, 4805–4810 (1996).
62. Chen, H., Beardsley, G. P. & Coen, D. M. Mechanism of ganciclovir-induced chain termination revealed by resistant viral polymerase mutants with reduced exonuclease activity. *Proc. Natl Acad. Sci. USA* **111**, 17462–17467 (2014).
63. Appleton, B. A., Loregian, A., Filman, D. J., Coen, D. M. & Hogle, J. M. The cytomegalovirus DNA polymerase subunit UL44 forms a C clamp-shaped dimer. *Mol. Cell* **15**, 233–244 (2004).
64. Pichlmair, A. et al. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. *Science* **314**, 997–1001 (2006).
65. Mi, H., Muruganujan, A., Ebert, D., Huang, X. & Thomas, P. D. PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools. *Nucleic Acids Res.* **47**, D419–D426 (2019).

Acknowledgements

We are grateful to Davide Gibellini for HIV FACS analysis, Han Chen for providing GST-UL54, and Jean Pesola for advice on statistical analysis. We thank Marcello Arsura for critically reviewing the manuscript. This research was supported by the European Commission under the Horizon2020 program (H2020-MSCA-ITN-2015) (S.L.), the Italian Ministry of Education, University and Research-MIUR (PRIN 20178ALPCM) (V. D.O.), "Cassa di Risparmio" Foundation of Turin (RF = 2019.2273) (V.D.O.), the Compagnia San Paolo of Turin (IFIBD) (M.D.A.), the AGING Project—Department of Excellence—Department of Translational Medicine, University of Piemonte Orientale (G.G.) and the University of Turin (RILO1801) (RILO1901) (S.L.) (M.D.A.) (V.D.O.) (F. G.) (M.B.). Work in the Thompson lab is funded by the National Institute of General Medical Sciences (R35GM118112) and National Institute of Health (F32GM128231). Work in the Coen lab was supported by the National Institute of Allergy and Infectious Diseases (R56AI019838 and R21AI141940). Work in the Pichlmair lab is funded by the ERC consolidator grant (ERC-CoG ProDAP, 817798) and the German Research Foundation (TRR179/TP11, TRR237/A07). Work in Stamminger lab is funded by the Deutsche Forschungsgemeinschaft (DFG) (STA357/7-1).

Author contributions

G.G., F.G., S.P., and S.L. conceived and designed the experiments. G.G., F.G., S.P., G.O., V.D.O., M.B., and V.G. performed the experiments and analyzed the data. A.J.S., P.R.T., E.W., S.M., and V.G. carried out the LC-MS/MS analyses. J.A.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. M.D.A., D.M.C., and A.P. critically revised the manuscript. 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 issued in October 20, 2019 ("PAD2 PER USO NELLA PREVENZIONE E/O TRATTAMENTO O DIAGNOSI DI INFEZIONI DA VIRUS DELLA FAMIGLIA HERPESVIRIDAE").

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-24178-6.

Correspondence and requests for materials should be addressed to M.D.A. or S.L.

Peer review information *Nature Communications* thanks the anonymous reviewers for their contribution to the peer review of this work.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021

Review

Human Cytomegalovirus and Autoimmune Diseases: Where Are We?

Francesca Gugliesi [1,†], Selina Pasquero [1,†], Gloria Griffante [2], Sara Scutera [1], Camilla Albano [1], Sergio Fernando Castillo Pacheco [1], Giuseppe Riva [3], Valentina Dell'Oste [1] and Matteo Biolatti [1,*]

1 Department of Public Health and Pediatric Sciences, University of Turin, 10126 Turin, Italy; francesca.gugliesi@unito.it (F.G.); selina.pasquero@unito.it (S.P.); sara.scutera@unito.it (S.S.); camilla.albano@unito.it (C.A.); sergiofernando.castillopacheco@unito.it (S.F.C.P.); valentina.delloste@unito.it (V.D.)

2 Department of Translational Medicine, Molecular Virology Unit, University of Piemonte Orientale Medical School, 28100 Novara, Italy; gloria.griffante@uniupo.it

3 Otorhinolaryngology Division, Department of Surgical Sciences, University of Turin, 10126 Turin, Italy; giuseppe.riva@unito.it

* Correspondence: matteo.biolatti@unito.it; Tel.: +39-011-670-5635

† These authors contributed equally to this work.

Abstract: Human cytomegalovirus (HCMV) is a ubiquitous double-stranded DNA virus belonging to the β-subgroup of the herpesvirus family. After the initial infection, the virus establishes latency in poorly differentiated myeloid precursors from where it can reactivate at later times to cause recurrences. In immunocompetent subjects, primary HCMV infection is usually asymptomatic, while in immunocompromised patients, HCMV infection can lead to severe, life-threatening diseases, whose clinical severity parallels the degree of immunosuppression. The existence of a strict interplay between HCMV and the immune system has led many to hypothesize that HCMV could also be involved in autoimmune diseases (ADs). Indeed, signs of active viral infection were later found in a variety of different ADs, such as rheumatological, neurological, enteric disorders, and metabolic diseases. In addition, HCMV infection has been frequently linked to increased production of autoantibodies, which play a driving role in AD progression, as observed in systemic lupus erythematosus (SLE) patients. Documented mechanisms of HCMV-associated autoimmunity include molecular mimicry, inflammation, and nonspecific B-cell activation. In this review, we summarize the available literature on the various ADs arising from or exacerbating upon HCMV infection, focusing on the potential role of HCMV-mediated immune activation at disease onset.

Keywords: human cytomegalovirus; autoimmunity; autoimmune diseases

Citation: Gugliesi, F.; Pasquero, S.; Griffante, G.; Scutera, S.; Albano, C.; Pacheco, S.F.C.; Riva, G.; Dell'Oste, V.; Biolatti, M. Human Cytomegalovirus and Autoimmune Diseases: Where Are We? *Viruses* **2021**, *13*, 260. https://doi.org/10.3390/v13020260

Academic Editors: Santo Landolfo and Pier Luigi Meroni

Received: 28 December 2020
Accepted: 5 February 2021
Published: 8 February 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

The adaptive immune response recognizes external pathogens as non-self antigens as opposed to the antigens from one's own body, known as self-antigens. Dysregulation of this response can lead to the failure to distinguish self from non-self antigens, a phenomenon known as immune tolerance, acquired during fetal development, responsible for a variety of autoimmune diseases (ADs) [1].

ADs result from a complex interaction between genetic predisposition and environmental factors [2–4], which trigger immune responses leading to tissue destruction.

ADs comprise a family of more than 80 chronic illnesses affecting approximately 3–5% of the general population [5,6]. The concordance of ADs in identical twins, consistently less than 100% (12–67%), highlights the importance of epigenetic and environmental factors and, especially, infections in AD pathogenesis [5,7].

Human cytomegalovirus (HCMV) is a ubiquitous virus belonging to the *Herpesviridae* family. HCMV displays a double strand (ds) DNA genome, characterized by an enormous genome capacity, with estimates of more than 200 open reading frames (ORFs), even though

ribosome profiling and transcript analysis detected additional previously unidentified ORFs (~751 translated ORFs) [8]. HCMV infection is lifelong in the host, due to virus ability to establish latency. Even though one well characterized viral reservoir is hematopoietic cells, the exact latency site remains still elusive. Interestingly, and contrary to the classical perspective, it is becoming evident that latency-associated gene expression mirrors lytic viral patterns, albeit at much lower levels of expression [9].

Nowadays, also epigenetic modifications emerged as critical players in the regulation of active/latent HCMV infection [10]. During latency, in infected $CD34^+$ progenitor cells and $CD14^+$ monocytes, HCMV chromatin is associated with repressive markers, such as H3K9Me3, H3K27Me3, and transcriptional repressors, like heterochromatin protein 1 (HP1) and the KRAB-associated protein 1 (KAP1) [11]. During myeloid differentiation and activation, transcriptional repressors are downregulated, and the viral chromatin carries transcriptional active markers such as acetylated histones (AcH) and phosphorylated histone H3 [11]. Several evidences suggest that HCMV chronic infection accelerates age-related epigenetic changes, pointing out the interplay between HCMV and epigenetic machinery regulation [12]. At the same time, epigenetic events play a pivotal role in the pathophysiology of autoimmune/inflammatory conditions [13]. To date, the exact correlation of HCMV epigenetic modifications and development of ADs is still missing, and studies addressing the impact of HCMV on epigenetic modification on AD's onset are required.

A large body of evidence has shown how HCMV can use several of its genes to manipulate the innate and adaptive immune system of the infected subject [14–19]. This feature alongside many others, such as its wide tropism [20–23], its ability to persist in the host during phases of latency and reactivation, and, as already mentioned, its global distribution [24], makes HCMV a candidate etiological agent of ADs. A causative link between HCMV infection and ADs may appear difficult to determine epidemiologically given the widespread prevalence of HCMV and the rare occurrence of ADs. Mounting evidence has increasingly associated HCMV infection with rheumatologic diseases—e.g., systemic lupus erythematosus (SLE), systemic sclerosis (SSc), and rheumatoid arthritis (RA)—and neurological disorders—e.g., multiple sclerosis (MS), enteric disorders, and metabolic disorders, such as type 1 diabetes (T1D).

Despite the great effort, researchers have not yet been able to discriminate whether HCMV is an initiator of AD or an epiphenomenon that may simply exacerbate the course of ADs. In this regard, multiple mechanisms have been proposed to explain HCMV-induced autoimmunity. Through a mechanism defined as "molecular mimicry", viral epitopes that are highly similar to host determinants may induce the development of antibodies that attack the self at the level of specific tissues, as it has been hypothesized for the viral tegument protein pp65 in SLE patients [25]. Intriguingly, upon HCMV infection, immunocompetent hosts tend to develop an autoimmune reaction through the generation of autoantibodies, which occurs more frequently in those individuals with a systemic involvement [26]. HCMV-infected bone marrow transplant recipients quite often develop organ-specific autoantibodies against the human aminopeptidase CD13 [27,28] or common phospholipid [29], whereas solid organ transplant recipients develop non-organ-specific autoantibodies [30]. Accordingly, hypergammaglobulinemia, cryoglobulinemia, and autoantibody production are common features of HCMV-driven mononucleosis [31,32]. This unspecific hyperactivation of humoral immunity is thought to represent a mechanism of viral immune evasion, because it curbs host B-cell responses. Once the tissue is infected, activated antigen-presenting cells (APCs) are attracted to the infection site and release high levels of cytokines and chemokines that activate autoreactive T- or B-cells, leading to loss of tolerance, a phenomenon called "bystander activation". Several pieces of evidence suggest a role of HCMV infection in vascular damage and stenosis [33,34], an event that is quite frequent and fatal in patients with ADs [35].

There is also some evidence indicating that HCMV infection and ADs mutually affect each other. In particular, while primary or secondary HCMV infection can induce chronic, systemic type I inflammation, which may promote autoimmunity, eventually leading to

ADs [36], autoimmune flares can also trigger HCMV reactivation [36]. HCMV-induced immunosuppression, which has severe consequences in transplant recipients, may also play a protective role in the course of ADs [37].

This review aims to provide an updated overview on the role of HCMV in the etiopathogenesis of ADs, focusing on the underlying mechanism that has been proposed for each specific disorder.

2. Modulation of the Immune System by HCMV

HCMV has established a complex relationship with the host immune system, for both systemic dissemination and latency [38]. Indeed, primary and latent HCMV infection can be kept in check by the host immune system in a hierarchical and redundant way through type I and II interferons (IFNs), natural killer cells (NKs), and $CD8^+$ and $CD4^+$ T-cells [16,17,38]. Conversely, in different clinical settings, patients become immunocompromised, and high systemic inflammatory response, particularly driven by cytokines such as TNF-α, as well as diminished immune function has been detected. The inflammatory cascades can stimulate the HCMV major immediate early promoter (MIEP), followed by HCMV reactivation from latency [38]. HCMV reactivation is also frequently observed in immunocompetent seropositive adults, where it may exacerbate chronic illnesses, such as ADs. Vice versa, the inflammatory environment of ADs, described in detail in the paragraphs below, may induce reactivation of HCMV, forcing replication.

HCMV, thanks to its continuous co-evolution with the host, has developed an arsenal of immune escape mechanisms to counteract the immune system, particularly the "unwanted" inflammation [38–41]. These "viral gambits" are discussed below.

Adaptive immunity is critical for the control of primary HCMV infections, which can later on be enhanced by clonal expansion of activated $CD4^+$ and $CD8^+$ T-cells [41]. To counteract this response, HCMV employs five viral glycoproteins (i.e., US2, US3, US6, US10, and US11), all capable of interfering with the presentation of the major histocompatibility (MHC) class I antigen [42] and the recognition of antigenic peptides by $CD8^+$ T-cells. Concurrently, an important role in regulating the production of antigenic peptides and inhibiting the production of viral epitopes [43] is played by HCMV miR-US4-1, which, by targeting the endoplasmic reticulum aminopeptidase 1 (ERAP1), inhibits the $CD8^+$ T-cell response. Likewise, HCMV miR-UL112-5p appears to downregulate ERAP1 expression, thereby inhibiting the processing and presentation of HCMV pp65 to cytotoxic T lymphocytes (CTLs) [43,44]. Finally, upon THP-1 cell infection, HCMV pUL8 reduces the levels of pro-inflammatory factors so as to inhibit inflammation [45], whereas HCMV pUL10 mediates immunosuppression by reducing T-cell proliferation and cytokine production [46].

On the other hand, innate immunity represents the host's first line of defense against external pathogens [47]. The initial intracellular response is triggered by pattern recognition receptors (PRRs), which after detecting pathogen-associated molecular patterns (PAMPs) can activate a downstream signaling pathway leading to the production of type I IFN and the release of pro-inflammatory cytokines. Also in this case, HCMV has devised different strategies to circumvent innate immunity [40,48,49]. For instance, our group has recently shown that the HCMV tegument protein pp65—also known as pUL83—binds to cyclic guanosine monophosphate–adenosine monophosphate synthase (cGAS), thereby inhibiting its ability to stimulate IFN-β production [50]. Similarly, the tegument protein UL31 has been shown to interact with cGAS, thereby decreasing cGAMP production and type I IFN gene expression [51].

Consistent with an immune escape function of HCMV tegument proteins, two studies by Fu et al. have shown that pp71—also known as pUL82—can inhibit trafficking of the stimulator of IFN genes (STING) [52], and that UL42 is a negative regulator of the cGAS/STING pathway [53]. Another HMCV glycoprotein, known as US9, can downregulate IFN type I by interfering with the mitochondrial antiviral-signaling protein (MAVS) and STING pathways [54]. Furthermore, the HCMV immediate–early (IE) 86 kDa protein

(IE86) downmodulates IFN-β mRNA expression by preventing nuclear factor-κB (NF-κB)-mediated transactivation of IFN-β [55]. Interestingly, a new study by Kim et al. [56] has revealed that IE86 may also inhibit IFN-β promoter activation by inducing STING degradation through the proteasome.

The innate immune system also relies on the concerted anti-microbial action of NKs, dendritic cells, and macrophages [47]. In particular, NKs play a primary role in counteracting viral infection thanks to their ability to recognize virus-infected cells through activating or inhibitory receptors—e.g., NKG2D and NKp30. As a consequence, HCMV has evolved various immune evasion strategies that rely on the modulation of NK receptors [57,58]. For example, HCMV UL142, UL148a, US9, US18, and US20 have all been shown to downregulate—to different extents and sometimes in an allelic-specific manner—MHC class I polypeptide-related sequence A (MICA), one of the eight different NKG2D ligands [59,60]. In addition, miR-UL112 and UL16 can both inhibit the expression of MHC class I polypeptide-related sequence B (MICB). Besides MICB, UL16 can also downmodulate the expression of UL16-binding protein 1 (ULBP1), ULBP2, and ULBP6 [61–64]. ULBP3 is instead targeted by UL142, which can also act as a MICA inhibitor [65,66]. The ability to concurrently evade multiple cellular pathways has also been shown for US18 and US20, both capable of inhibiting MICA and the NKp30 ligand B7-H6 [67,68] (Figure 1).

Moreover, HCMV encodes a set of Fcγ binding glycoproteins (viral FcγRs, vFcγRs) that bind to the Fc region of host IgG and facilitate evasion from the host immune response [69]. Particularly four vFcγRs encoded by HCMV have been identified: gp68 (UL119-118), gp34 (RL11), gp95 (RL12), and gpRL13 (RL13) [70–73]. They are crucial for viral escape from both innate and adaptive immune responses, including antibody dependent cellular cytotoxicity (ADCC) [71].

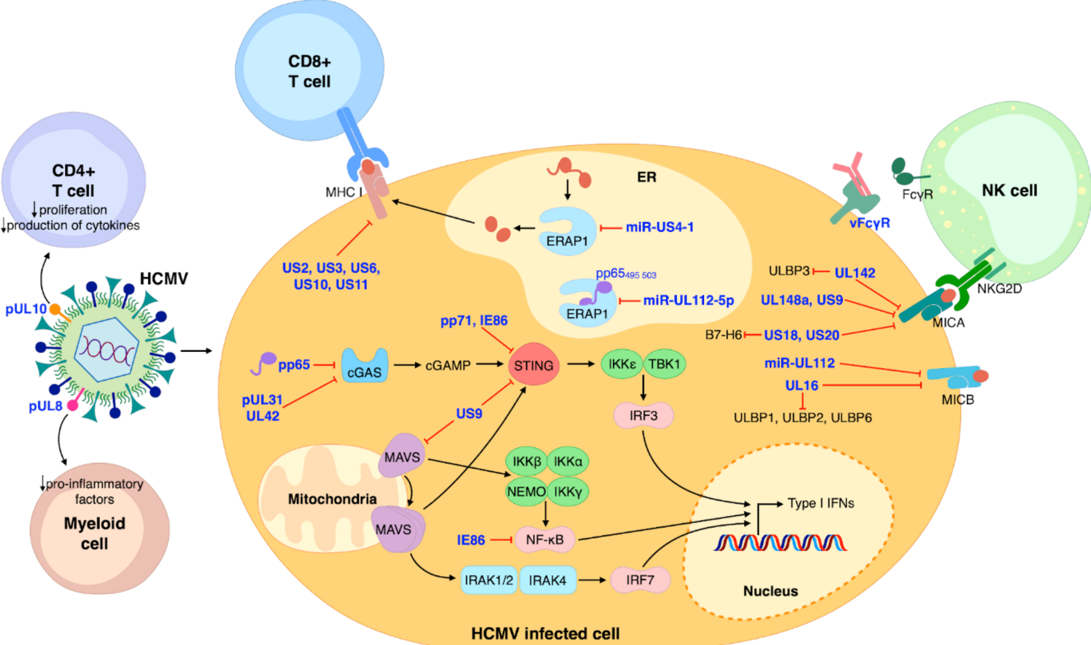


Figure 1. Schematic model summarizing the major aspects of HCMV modulation of the immune system. NK cells, CD8+ and CD4+ T-cells, and myeloid cells are the main protagonists of host immune control of HCMV infection. HCMV proteins are represented in blue. Black arrows indicate stimulation/activation; red lines represent inhibition.

Another strategy that HCMV has acquired is the ability to produce viral products homologs to cytokines, chemokines, and their receptors, which can alter the immune response and the clearance of the virus during the productive or the latent phase of the infection [15]. Among these factors, HCMV encodes an interleukin 10 (IL-10) homolog, known as cmvIL-10, which can modulate the immune response and induce replication and

persistence of the virus. cmvIL-10 can stimulate the differentiation of autoreactive B cells on one hand and on the other hand suppress pro-inflammatory factors, tilting the immune response and inducing a chronic productive infection. In different autoimmune disorders, IL-10 presents an altered expression due to polymorphisms in its promoter, and elevated levels of IL-10 have been detected in SLE and Sjögren's syndrome (SS) patients [74–76]. Although a direct relationship between HCMV, IL-10, and autoimmune disorders has not yet been recognized, further investigations are needed to better clarify a possible role of HCMV cytokine homologs in these diseases.

Interestingly, polymorphisms in cytokine signaling pathways might be involved in autoimmune disorders in association with viral infection. For example, the association between genetic polymorphisms related to cytokines, as single-nucleotide polymorphisms (SNPs) in signal transducer and activator of transcription 4 (STAT4) or interleukin 10 (IL-10), and different autoimmune disorders has been described [77–79], identifying IFN-α as an environmental modifier of the STAT4 risk allele and indicating a major risk to develop the disorder during a viral infection [80]. These results suggest that an altered function or expression of different cytokines can predispose to the autoimmune disease or modulate the disease manifestations.

3. Documented Mechanisms of HCMV-Induced Autoimmunity

HCMV can induce or perpetuate autoimmunity through different processes that can be divided into two categories: (1) antigen-specific (i.e., molecular mimicry) and (2) non-antigen-specific (i.e., bystander activation). From an immunopathological perspective, HCMV can trigger or sustain autoimmunity through the following three mechanisms: (i) autoantibodies production, (ii) enhanced inflammation, and (iii) vascular damage (Figure 2). These will be further discussed below.

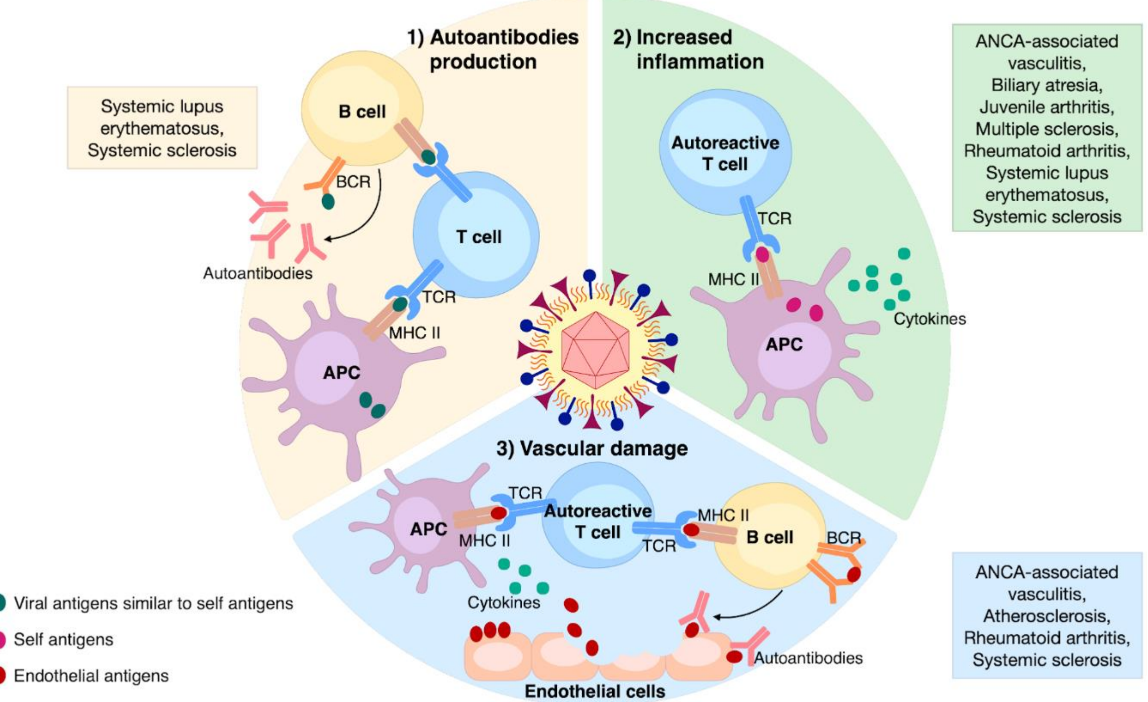


Figure 2. The main mechanisms involved in HCMV-induced autoimmunity and associated ADs. (**1**) Autoantibodies production: the occurrence of viral epitopes, structurally similar to self-ones, can induce the activation of both T- and B-cells through their presentation by APCs; (**2**) increased inflammation: non-specific anti-HCMV immune response leads to the release of self-antigens and cytokines from the affected tissue; those self-antigens presented by APCs can stimulate autoreactive T-cells; (**3**) vascular damage: enduring HCMV infection triggers vascular damaging; the release of endothelial antigens and cytokines induces the activation of autoreactive T-cells and B-cells, culminating in aggression of endothelial cells via specific autoantibodies.

3.1. Autoantibodies Production

This particularly harmful effect of HCMV is due to viral-induced molecular mimicry, which is a mechanism through which HCMV infection activates T-cells that are cross-reactive with self-antigens. Although among the *Herpesviridae* family, the Epstein–Barr virus (EBV) has been more extensively studied in this regard [81], HCMV has also been frequently involved in the generation of cross-reactive autoantibody in ADs. For example, patients affected by SSc express different autoantibodies able to recognize both cellular proteins and their homologous HCMV counterparts—e.g., anti-topoisomerase I/HCMV pUL70 [82] and anti-cell surface integrin–neuroblastoma-amplified gene (NAG)-2/HCMV pUL94 [83] antibodies. The association of HCMV infection with ADs does not appear to be solely restricted to SSc given that SLE patients can also express high levels of two anti-pp65 and anti-pp150 antibodies [25,84,85]. Consistent with a role of HCMV pp65 in autoimmunity, immunization of BALB/c mice with peptides derived from the C-terminus of this viral protein led to the generation of anti-dsDNA and antinuclear antibodies, inducing severe signs of glomerulonephritis [86]. More recently, SLE patients were also found to express high levels of IgG antibodies against the HCMV DNA-binding nuclear protein UL44 [87]. Intriguingly, this antibody was able to co-immunoprecipitate UL44 and nuclear SLE autoantigens during virus-induced apoptosis, suggesting a novel contribution of HCMV to humoral immunity in ADs. Other possible associations of antibodies against HCMV structures and self-antigens were speculated but not confirmed in other ADs [88,89].

Humoral autoimmunity can also be induced by non-specific B-cell activation, since HCMV can be considered a bona fide polyclonal B-cell activator. In this regard, HCMV can induce B-cell proliferation and favor autoantibody production by interacting with Toll-like receptor (TLR)7/9 in plasmacytoid dendritic cells (pDCs) [90]. More recently, cross-talk between B-cell-activating factor (BAFF) and TLR9 signaling has been shown to promote IgG secretion and survival of B-cells following HCMV infection [91].

3.2. Enhanced Inflammation

The mechanism behind this nonspecific antiviral immune response is best known as bystander activation, defined as the stimulation of autoreactive T-cells by self-antigens presented by APCs. The presence of terminally differentiated $CD4^{+}CD28^{-}$ T-cells is typical of HCMV-infected individuals [92,93], including patients with ADs, such as RA [94]. Reactivation and replication of HCMV in inflamed tissue has been found to induce T-cell differentiation of the pathogenic and dysregulated $CD4^{+}CD28^{-}$ subset under autoimmune conditions, albeit these cells do not seem to have a direct auto aggressive behavior, as described in detail by Bano et al. [95]. In this review, the authors also speculate that RA-infected synovial fibroblasts may directly or indirectly—through the release of non-infectious exosomes—present HCMV antigens to T-cells, thereby inducing their terminal differentiation. This hypothesis has been recently substantiated by a proof of concept study showing that, in antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), the expansion of $CD28^{-}$ T-cells was reduced by an antiviral therapy able to suppress HCMV subclinical reactivation, indicating that expansion of this clone was HCMV-dependent [96]. By contrast, Wu et al. [97] have more recently shown that the expansion of $CD4^{+}$ $CD28^{-}$ cells in SLE patients is negatively associated with disease activity—lupus low disease activity state is associated with lower anti-DNA levels—and that the polyfunctional $CD8^{+}$ T-cell response to HCMV pp65 is not impaired. Moreover, HCMV seropositive MS patients displayed not only an altered B-cell phenotype and function, but also a modulation of the IFNβ response and a reduced pro-inflammatory cytokine B-cell profile, indicating a putative protective role of HCMV [98].

In ADs characterized by high levels of inflammation and chronic immune stimulation, such as RA, a causative role of HCMV has also been hypothesized. For instance, after specific HCMVpp65 long-term stimulation, increased anti-HCMV IgG antibodies and intracellular IFN-γ-producing HCMVpp65-specific $CD28^{-}CD8^{+}$ T-cells were observed in RA and juvenile arthritis (JIA) patients vs. healthy controls (HCs), indicating a possible

enhancement of the inflammatory response following endogenous HCMV reactivation [99]. Moreover, an increased proportion of terminally differentiated immunoglobulin-like receptor 1 (LIR-1^+) $CD8^+$ T-cells was detected in HCMV seropositive RA patients. These cells were characterized by cytolytic activity, pro-inflammatory properties, and anti-infectious effector features, all distinctive characteristics of the so-called "chronic infection phenotype", probably involved in the inflammatory pathogenesis of RA [100].

A cause–effect relationship between HCMV infection and other systemic ADs, such as SLE and SSc, is supported by experiments testing the in vitro response to the HCMV antigen in T-cells from SLE and SSc patients. The enhanced expression levels of IFN-γ, IL-4, and IL-2 as well as the increased number of memory T-cells found in these patients led, in fact, the authors to conclude that exposure to HCMV may promote fibrosis and vascular damage [101].

In recent years, Arcangeletti and co-workers have taken a closer look at the interplay between HCMV and the immune response in SSc and inflammation. Interestingly, in HCMV-infected human dermal fibroblasts, this group was able to detect increased HCMV-specific $CD8^+$ T-cell responses associated with disease parameters, which were paralleled by enhanced expression of several fibrosis- and apoptosis-associated factors involved in SSc pathogenesis [102,103].

HCMV can amplify inflammation through other mechanisms. For instance, the latency-associated gene US31 is expressed at higher levels in PBMCs from SLE patients vs. HCs. This upregulation may be relevant to AD pathogenesis, because US31, by acting through the non-canonical NF-κB pathway (NF-κB2), can alter the immunological properties of monocytes and macrophages and promote an M1 inflammatory phenotype [104].

With regard to the interplay between HCMV and MS, murine cytomegalovirus (MCMV) can promote EAE in resistant BALB/c mice by activating inflammatory APCs and $CD8^+$ encephalitogenic-specific T-cells and promoting the M1 phenotype of microglia [105].

Biliary atresia (BA), classified as an autoimmune-mediated disease, is a disorder characterized by inflammation, fibrosis, and obstruction of the bile duct. To simulate BA, mice depleted of Treg cells were infected with low doses of MCMV, a condition that led to increased expression of IFN-γ-activated genes and inflammation, attesting an involvement of CMV in disease progression [106].

NKs play a crucial role in homeostasis and immune responses. Besides exerting a cytotoxic effect, NK activation can trigger the release of different pro-inflammatory cytokines, promoting excessive inflammation, which eventually leads to ADs. In this regard, distinct NK subsets are capable of reaching different tissues where they can exert a protective effect on immune homeostasis. Such an example is the expansion of adaptive $NKG2C^+$ cells in acute HCMV infection or reactivation, inducing a protective effect [107]. Furthermore, higher percentages and absolute numbers of these cells are found in MS patients positive for HCMV, again indicating that HCMV may play a protective role in this autoimmune condition [108]. On the other hand, a study by Liu et al. revealed the existence of an antibody able to recognize HCMV pp150 across various ADs. The fact that this antibody was also able to recognize the single-pass membrane protein CIP2A and promoted cell death of $CD56^{bright}$ NKs, a subset whose expansion is frequently observed in autoimmunity, led to the conclusion that the generation of HCMV-induced autoantibodies may be responsible for the onset of ADs [85].

Unconventional $\gamma\delta$ T-cells are potent inducers of cytotoxicity and have been recently identified as determinants of adaptive immunity against pathogens and tumors via APC activation and stimulation of other leukocytes [109]. Once activated, they trigger tissue repair, inflammation, and lysis of different cell types. In patients affected by the severe combined immunodeficiencies (SCID), an increase in $\gamma\delta$ T-cells associated with HCMV infection and autoimmune cytopenia was observed, suggesting that HCMV may promote expansions of these cells [110]. However, the direct involvement of HCMV in the activation of $\gamma\delta$ T-cells, as well as the direct role of these cells in ADs, has yet to be clarified.

3.3. Vascular Damage

HCMV plays an important role in vascular damage through endothelial cell (EC) apoptosis, infiltration of inflammatory cells, and smooth muscle cell proliferation. Lunardi and co-workers were the first to uncover a correlation between HCMV infection and endothelial damage in SSc [111]. The mechanism of HCMV-induced vascular damage was later linked to molecular mimicry characterized by auto aggression of ECs through release of specific autoantibodies against NAG-2/UL94 proteins, as described in Section 3.1. Indeed, the immunization of BALB/c mice with UL94 and NAG-2 peptides coupled with a carrier protein caused ischemic lesions on footpads and tails. Moreover, treatment of ECs with the same antibodies resulted in increased reactive oxygen species (ROS) production [33].

In atherosclerosis, an auto-inflammatory disorder with an autoimmune setting, HSP60 autoantibodies, which share homology with UL122 and US28 HCMV peptides, have been reported. These peptides present sequence homology also with different EC surface molecules [112]. DNA microarray-based experiments showed that these purified anti-HCMV antibodies can modulate the expression of various molecules (e.g., adhesion molecules, chemokines, molecules involved in inflammation, etc.) involved in EC activation and damage [113].

Finally, HCMV infection has been positively associated with $CD4^{+}CD28^{-}$ T-cell expansion and high cardiovascular disease (CVD) mortality risk among RA patients, further confirming a direct causal link between HCMV and vascular damage in AD [114,115]. The expansion of $CD4^{+}CD28^{-}$ T-cells in HCMV-positive/ANCA-associated vasculitis (AAV) patients, expressing a Th1 phenotype, with high levels of IFN-γ and TNF-α production and co-expression of different endothelial homing markers [96], further corroborates the role of HCMV in inducing AD-related vascular damage.

4. The Main Autoimmune Diseases Associated with HCMV Infection

4.1. Rheumatologic Diseases

4.1.1. Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a chronic AD characterized by connective tissue inflammation and heterogeneous clinical manifestations, ranging from mere cutaneous and musculoskeletal features to kidney and/or central nervous system involvement, often associated with significant morbidity and mortality. Although the causes of SLE are not clearly understood, many have proposed that SLE may be due to a combination of genetic predisposition and environmental factors (e.g., UV exposure, infection, and stress) [116,117].

All SLE patients inevitably show abnormalities in monocytic lineage cells, which can lead to T-cell deficiencies, polyclonal B-cell activation, immune complex formation, and autoantibody production. In this regard, the peculiar ability of HCMV to establish lifetime latency and to periodically shift between the lytic and latent stage has been linked to the aberrant humoral response in SLE. Fittingly, augmented anti-HCMV IgM/IgG titer tends to correlate with clinical and immunological manifestations of SLE [118]. Studies that found an association between HCMV and SLE disease were often performed in European countries [119,120]. Additionally, differences in the prevalence of HCMV infection in SLE patients were reported by different research groups. For example, Takizawa et al. [121] found that 149 of 151 patients with rheumatologic disease were infected by HCMV, by pp65 antigenemia assay, and all 74 SLE patients were positive for HCMV infection. Newkirk et al. [122] found that the prevalence of HCMV infection in SLE patients was 60% by using ELISA kits to detect HCMV specific antibodies. After adjusting for the rheumatoid factor, Su et al. [123] found that 84 of 87 SLE patients (96.55%) were HCMV IgG-positive, and that nine (10.34%) were HCMV IgM-positive. On the other hand, several other studies did not observe a direct association between HCMV seroprevalence and SLE [124–126]. For examples, James et al. reported that HCMV infection was not related

to SLE [126]. Altogether, these results suggest that to date we do not have a complete understanding of the relationship between HCMV infection and SLE development.

A potential role of HCMV in SLE pathogenesis was initially proposed by several groups after identifying specific autoantigens induced upon HCMV infection [89,122]. Molecular mimicry has been described also for another member of the *Herpesviridae* family, i.e., EBV, that may be involved in the pathogenesis of SLE. Indeed, anti-Epstein–Barr nuclear antigen 1 (EBNA1) antibodies can recognize human proteins such as SmB and Ro60 [127].

As already mentioned, (see HCMV pp65, Section 3), HCMV can lead to the production of autoantibodies against nuclear proteins, such as in the case of the LA protein. Specifically, HCMV can directly—or indirectly, through molecular mimicry—induce cell surface expression of this small nuclear ribonucleoprotein, thereby leading to the production of autoantibodies in genetically susceptible individuals [122,128]. Subsequently, two independent groups [25,86] showed that immunization of previously non-autoimmune mice with peptides encompassing the HCMV epitope $pp65_{422–439}$ led to the appearance of autoantibodies against nuclear components while inducing early signs of nephritis resembling human SLE. Importantly, high levels of serum anti-$pp65_{422–439}$ antibodies were found in patients with SLE, suggesting that pp65 contained B-cell epitope(s) that could trigger autoimmunity in genetically predisposed individuals [25]. The same authors uncovered amino acid sequence homology between HCMV $pp65_{422–439}$ and the TATA-box binding protein associated factor 9 ($TAF9_{134–144}$) and detected the presence of specific antibodies against these epitopes in association with anti-nuclear and anti-dsDNA antibodies, typically found in SLE, alongside increased anti-TAF9 antibodies in sera from SLE patients [86].

More recently, Neo et al. have described a potential alternative process involving UL44, a DNA-binding phosphoprotein essential for HCMV DNA replication [87]. The observation that after translocation to the nucleus, UL44 interacted with other viral and host proteins to increase viral DNA replication efficiency led these authors to hypothesize that delayed clearance of apoptotic cellular material in genetically predisposed individuals may favor the presentation of intracellular self-antigens to humoral immunity. They succeeded in isolating a human UL44 antibody from the sera of SLE/HCMV IgG seropositive patients, showing that it could bind to UL44 complexed with cell-surface localized SLE autoantigens during virus-induced apoptosis. Thus, based on these findings, it is conceivable that HCMV may trigger and/or potentiate the host humoral immune response to nuclear self-antigens, predisposing infected individuals to SLE.

Genome-wide association studies (GWAS) have identified over 50 susceptibility loci for SLE in the population (mostly genes regulatory regions). Therefore, it is crucial to investigate the link between genetic susceptibility and viral infections in the development of SLE. For example, Harley and colleagues demonstrated that EBV gene products that serve as transcription factors have preferential interaction with loci containing risk alleles [127]. However, if any of the HCMV proteins preferentially bind SLE risk loci is still to be addressed.

Under a clinical point of view, dysfunction of the immune system has been long known to increase the risk of infection among SLE patients, accounting for approximately 50% of hospitalizations during the course of the disease [129], suggesting that a lifelong immunosuppression of an individual, as it is often the case for SLE patients, may favor HCMV reactivation [130]. This hypothesis was later on corroborated by findings from a 26-year retrospective study showing that infections, including those caused by HCMV, were amongst the top three causes of death in SLE patients [131], raising the important question of which risk factors are associated with HCMV disease in the SLE population. This question has been recently answered by a systematic review [132] identifying the following risk factors: (i) high viral load, which together with enhanced levels of HCMV antigenemia correlated with the development of life-threatening end-organ damage; (ii) lymphopenia, resulting in failure to mount a host cellular immune response against HCMV; and (iii) type

of treatment—HCMV disease progression correlated with higher corticosteroid doses and/or immunosuppressants [133].

HCMV infection is also known to trigger SLE flares through direct cytopathic effects and/or activation of inflammatory processes, thus causing both systemic and organ-specific disease. Lastly, the clinical features of HCMV infection themselves happen to mimic SLE flares, further complicating the clinical picture of SLE patients [134].

4.1.2. Systemic Sclerosis

Systemic sclerosis (SSc) is a chronic systemic inflammatory disease characterized by vasculopathy and extensive fibrosis. It has the highest mortality among ADs due to pulmonary hypertension and lung fibrosis. The etiology still remains unknown, although genetic predisposition, environmental factors, and infectious agents have all been considered as potential triggering factors [135–137]. The activation of the immune system plays a key role in SSc pathogenesis and is probably the link between initial vascular involvement and the end-stage of the disease (i.e., tissue fibrosis), raising the hypothesis that certain autoantibodies may not simply be epiphenomena but rather play a central role in disease pathogenesis. In particular, intracellular antigens autoantibodies have been associated with specific SSc subsets [138], whereas cell surface antigens autoantibodies production has been shown to cause EC damage and apoptosis and activation of fibroblasts, T lymphocytes, and macrophages. In turn, these activated cells tend to secrete higher levels of cytokines, leading to changes in the extracellular matrix, one of the hallmarks of SSc.

Among infective agents, herpesviruses have been suggested to be causative agents in the immunopathogenesis of SSc [139,140]. Indeed, antibodies against HCMV and EBV are more frequently detected in SSc than in healthy controls [141–143].

The statistically significant association with HCMV infection in Swiss SSc patients (59% seropositivity in SSc patients compared with 12–21% controls) [144] has not been observed in other studies so far [143,145], even though higher HCMV antibody concentrations have been found in SSc patients [146,147]. In this regard, future studies should clarify why a ubiquitous virus such as HCMV only triggers an autoimmune response in certain individuals, whereas in others it has no effect.

HCMV can maintain an active, persistent replication for the life span of the immunocompetent host, particularly thanks to its macrophage and endothelial tropism [148]. Starting from the observation that HCMV antibodies are prevalent in SSc patients [144] and that UL70 viral protein can be recognized by anti-topoisomerase I antibody, Lunardi et al. were the first to propose a novel pathogenesis mechanism of SSc based on HCMV molecular mimicry of the cellular protein NAG-2 expressed on ECs and fibroblasts, with the latter being involved in the so-called "scleroderma like phenotype" linked to SSc pathogenesis [33,83,111] (see Sections 3.1 and 3.3).

The most frequently found autoantibodies among SSc patients are those directed against centromere proteins (anti-CENPs), DNA topoisomerase I (anti-topo I), and RNA polymerase III (anti-RNA polIII). Of note, in SSc, there is a significant correlation between the expression of autoantibodies against RNA polIII and the presence of specific clinical features, such as high risk of diffuse cutaneous disease, short survival time, and renal involvement. Moreover, SSc patients expressing autoantibodies against anti-topo I are at high risk of developing pulmonary interstitial fibrosis, whereas patients with CENP autoantibodies have the best prognosis [138]. Lastly, a recent study [149] evaluating the relationship between the immune response of SSc patients to six major antigens of HCMV (i.e., UL57, UL83, UL55, UL44, p38, and UL99) and specific clinical and immunological characteristics of the disease found that the presence of anti-UL44 antibodies correlates with arthritis, a clinical feature of SSc. This finding supports the idea that anti HCMV antibodies may play an important role in breaking tolerance and triggering SSc pathogenesis.

4.1.3. Rheumatoid Arthritis

In spite of an increasing body of evidence, a functional role of HCMV in the pathogenesis of rheumatoid arthritis (RA) has not yet been conclusively proven due to controversial findings, whereas a correlation between EBV and RA has already been found. There are several examples of molecular mimicry between EBV and self-antigens relevant to RA, such as HLA-DRB1 polymorphisms, human interleukin (h IL)-10, and a CXC chemochine receptors [150]. While some early studies found high HCMV seroprevalence among RA patients [120,151,152], other investigations could not establish a clear association between HCMV infection and RA [144,153–155]. In support of a role of HCMV in RA, some authors have more recently reported the presence of HCMV replication in synovial specimens from RA patients, which correlated with increased disease severity, revealing a higher incidence of HCMV infection in RA patients than previously thought [152,156]. However, the fact that immunosuppressive therapy can lead to HCMV reactivation does not allow drawing any definitive conclusions as to whether HCMV may be involved in RA initiation rather than its exacerbation.

The term "rheumatoid arthritis" was defined in 1859 by Alfred Baring Garrod to distinguish this chronic systemic autoimmune disease from other forms of arthritis (e.g., osteoarthritis, spondyloarthritis, etc.) [157]. RA affects 0.5–1% of the worldwide population, with higher prevalence in the elderly [158], with a female to male ratio of 3:1 [159]. RA is a T-cell-driven autoimmune disease, accompanied by autoantibody production that affects primarily the lining of the synovial joints, leading to destructive synovitis, progressive disability, and even to premature death due to extra-articular manifestations, such as vasculitis [160,161]. The chronic inflammation and subsequent tissue damage of the joints is caused by the deposition of immune complexes (ICs) composed of autoantibodies bound to their cognate autoantigens, which attract innate immune cells to the site of deposition, with subsequent release of proteolytic enzymes, slowly degrading the synovial tissue in an endless vicious cycle [160]. Autoantibodies isolated from patients with RA were shown to recognize citrullinated proteins (anti-citrullinated peptide antibodies, ACPAs) and IgG (rheumatoid factor, RF) [162]. Interestingly, these autoantibodies were found to be already present in a subset of RA patients years before the disease onset and could predict a more aggressive and severe progression [163,164].

Citrullination is a post-translational modification catalyzed by a family of peptidylarginine deiminases (PADs) that convert peptidylarginine into peptidylcitrulline, whose aberrant dysregulation has been linked to several inflammatory conditions, such as ADs, cancer, and neurodegenerative diseases [165–169]. The theory that citrullination is involved in the etiopathogenesis of RA has been supported by several lines of evidence [170–173], but the mechanisms that trigger citrullination and, therefore, initiate RA development are still unknown. Interestingly, many genetic and environmental factors have been associated with RA pathogenesis, especially among ACPA-positive patients. According to the so-called "two hit" model, in genetically predisposed individuals, the first hit is represented by environmental triggers, such as smoking or infection, which induce citrullination of peptides that are successively presented to autoreactive T-cells, leading to the generation of high-affinity anti-citrullinated peptide antibodies. These events are thought to occur years before the onset of the disease. During the second hit, synovitis and further citrullination together with pre-existing ACPA lead to the development of chronic inflammation due to persistent formation of ICs [174]. Intriguingly, three independent studies [175–177] have shown that citrullination of EBV proteins may create epitopes that are recognized by ACPA isolated from RA patients, indicating that ACPAs can indeed react with a viral deiminated protein and suggesting that herpes viruses, such as EBV, are environmental factors contributing to the onset and/or development of RA. Due to the lack of direct evidence, we cannot however make a similar claim about HCMV species. In this regard, it would be interesting to investigate whether viral infections are directly involved in PAD activation and whether subsequent citrullination of cellular and/or viral proteins is dysregulated in AD. Very recently, Casanova et al. [178] have reported citrullination of human cathelicidin

LL37, a host defense peptide, in human rhinovirus (HRV)-infected bronchial epithelial cells, which negatively affects the antimicrobial and antiviral activity of this peptide, suggesting that citrullination may constitute a viral immune evasion mechanism.

On the other hand, an immune response to latent HCMV has been shown play a critical role in the progression of inflammation and structural damage of joints in RA patients [179]. In this regard, it is important to point out that RA patients tend to display expansion of a particular subset of T-cells $CD4^+$ lacking the costimulatory molecule CD28, required for T-cell activation and survival [180,181] (see Sections 3.2 and 3.3). Intriguingly, the frequency rate of this clonal expansion, which rarely exceeds 1% in the elderly, quite often reaches values between 5% and 10% in RA patients, where it is associated with extra-articular manifestations, such as early atherosclerotic vessel damage [94], probably due to the ability of $CD4^+CD28^-$ T-cells to exert a cytotoxic activity and directly attack the vascular tissue [182]. As it correlates with disease severity and the extent of extra-articular involvement, the frequency rate of $CD4^+CD28^-$ T-cells in RA has been proposed to be a predictor of future acute coronary events. Intriguingly, HCMV infection is a major trigger of $CD4^+CD28^-$ T-cells expansion [92]. The fact that these T-cells are only found in HCMV-positive RA patients and respond to HCMV antigen stimulation in vitro suggests that HCMV infection contributes to increased inflammation and RA aggravation by accelerating extra manifestations, such as coronary damage. The detection of $CD4^+CD28^-$ T-cells in other inflammatory conditions, such as psoriatic arthritis, MS, inflammatory bowel diseases (IBDs), cardiovascular diseases, chronic rejection, ankylosing spondylitis, and Wegener's granulomatosis, has led to the hypothesis that HCMV-mediate induction of $CD4^+CD28^-$ T-cells may be a shared mechanism of ADs [92,93,183,184]. Eventually, $CD4^+CD28^-$ T-cells may respond to autoantigens in the synovium and produce cytotoxic molecules or activate macrophages to release pro-inflammatory cytokines that leads to cartilage erosion [95]. As already mentioned, HCMV DNA, specific antigens, and infectious virus particles have all been detected in synovial tissue and fluid from the joints of 10% to 50% RA patients [156,185–188]. Interestingly, HCMV has been associated with a significantly increased risk of cardiovascular disease also in non-RA patients [189–191], which is not so surprising in light of mounting evidence supporting the ability of HCMV to manipulate the host cell metabolism to favor viral growth [192].

Increased RA disease activities in HCMV-seropositive individuals may also be linked to the expansion of another specific of $CD8^+$ T-cell subset, which preferentially expresses the inhibitory NK cell receptor LIR-1 and exerts a cytolytic effect [100]. Indeed, expression of LIR-1 on $CD8^+$ T-cells is upregulated following HCMV infection [193] and results in reduced T-cell proliferation [194]. LIR-1 is also considered a marker of premature immune senescence, since its upregulation may limit tissue damage otherwise caused by persistent anti-HCMV immune response [195].

In conclusion, emerging evidence indicates that HCMV may contribute to the development of RA by exacerbating and/or accelerating disease severity, especially in patients with vascular manifestations. However, there is disagreement on whether HCMV infection is an initiating event or just an epiphenomenon.

4.2. Neurological Diseases

Multiple Sclerosis

Multiple sclerosis (MS) is a chronic autoimmune inflammatory disease affecting the central nervous system (CNS) characterized by the destruction of neuronal axonal myelin. It mainly affects young adults, with a higher prevalence in females, often leading to non-traumatic neurological disabilities. The progressive deterioration of motor, sensory, and cognitive functions is characterized by specific histopathological markers, such as demyelination, leukocyte infiltration, neurodegeneration, and reactive gliosis of the CNS [196]. Although the precise etiology of MS is not yet clear, it is thought to occur in genetically susceptible individuals following interaction with one or more environmental factors. The most common environmental risk factors are sunlight exposure, vitamin D levels, cigarette

smoke, and infectious agents [197]. In particular, several epidemiological studies have reported a significant association of herpesvirus infections with MS pathogenesis. Among herpesviruses, EBV, which infects about 95% of the global adult population, has often been proposed as the major culprit candidate [198,199]. Although no other pathogens have been as strongly associated with MS as EBV, many studies have looked at a possible correlation between MS susceptibility and infection with other herpesviruses, in particular HCMV. One of the peculiarities of HCMV is that of being able to establish a permanent latent infection whose prevalence appears to be inversely related to the socioeconomic development of the population in question—in good agreement with the broader "hygiene hypothesis", according to which the correlation between HCMV and MS may be indirectly linked to exposure to other environmental factors [200]. Contrary to this assumption, others have proposed that the immunopathology of MS can in fact be influenced by HCMV, as the impact of this latter on the immune system ultimately interferes with the host immune response to other pathogens (i.e., heterologous immunity) [201].

With regard to molecular evidence supporting a relationship between MS and HCMV infection, two different studies found higher HCMV DNA loads in a cohort of MS patients compared to HCs [202,203]. Moreover, the same authors detected positivity for anti-HCMV IgG antibodies in almost 80% of the MS patients examined. However, the fact that there were no significant differences in anti-HCMV antibody concentration between MS patients and HCs led the authors to conclude that the presence of these antibodies alone was not a significant marker for MS development. Finally, the hypothesis that the risk of developing MS increases due to systemic HCMV infection is also supported by some MS cases where opportunistic reactivation of HCMV infection has been linked to worsening of pre-existing MS [204,205].

By contrast, other studies have shown a negative correlation between the development of MS and HCMV seropositivity [200,201], although skeptics argue that this may not be the result of a direct protective effect but simply an epiphenomenon related to the adoption of a Western lifestyle or to early viral infections. In this regard, Alari-Pahissa and colleagues [200] conducted a study aimed to determine whether the serological status of HCMV in early MS patients was different from that observed in non-early MS patients, in particular by looking at the putative association of this virus with the clinical course of the disease and the humoral immune response against other herpesviruses. In a nutshell, the authors found that HCMV increased not only the production of pro-inflammatory cytokines (e.g., TNF-α and IFN-γ) but also the antibody-dependent cellular cytotoxicity mediated by adaptive NKs, an activity that is known to influence the host immune response to other pathogens [206,207]. Since anti-EBNA-1 antibody levels had been previously shown to directly correlate with increased MS disease activity [208], the authors asked whether they could establish an association between a specific humoral response in MS patients and HCMV positivity. Interestingly, they observed a decrease in the EBNA-1 index related to disease duration in HCMV-positive MS patients aged 40 years or younger [200,209]. Moreover, the same patients displayed an increased proportion of end-differentiating T-cells. Thus, altogether these findings indicate that HCMV seropositive individuals close to MS onset tend to develop an inflammatory process involving a pool of more differentiated T-cells with respect to HCMV seronegative individuals. In this setting, persistent HCMV infection might divert immunological resources, reducing the risk of autoimmunity, in line with the hypothesis that it may be protective for MS development. A more recent study has recorded lower anti-HCMV IgG seroprevalence rates in MS patients—either younger or older than 40 years—compared to HCs [209]. Of note, these patients had relapsing MS and were not subjected to any steroid or disease-modifying treatments at the time of sampling. Overall, these findings indicate that, in MS patients, HCMV infection not only modulates the immune response by reducing the severity of the disease, but may also affect the response against EBV infection.

A very recent study has instead examined the possibility that HCMV may also induce changes in the peripheral B-cell compartment in MS patients. Both B-cell phenotype

and function were found to be influenced by HCMV infection, promoting early stages of differentiation in relapsing–remitting MS (RRMS) and reducing the pro-inflammatory cytokine profile in advanced MS. Overall, the results of this study argue in favor of the hypothesis that HCMV infection modulates B-cell subset distribution and IFN-β response in MS patients. Furthermore, they indicate that HCMV infection is associated with a reduced pro-inflammatory cytokine profile in progressive MS (PMS), thereby providing mechanistic insights into the alleged protective action of HCMV in MS [98].

In conclusion, the relationships and associations of HCMV infection with the development and progression of MS appear physiologically relevant and, thus, worthy of further investigation. Even though it is currently difficult to say with any certainty whether HCMV exerts a beneficial or harmful effect on MS, the latest findings seem to concur that there is a correlation between HCMV infection and a lower susceptibility to MS.

4.3. Enteropathies

In recent years, the role of HCMV in the pathogenesis of gastrointestinal diseases has gained increasing attention. A large body of literature has in fact documented that epithelial cells of the intestinal mucosa are the primary sites of HCMV replication both in vivo [210] and in vitro [211,212]. Moreover, HCMV has also been pinpointed as the main cause of graft failure after intestinal/multivisceral transplantation [213,214].

Among autoimmune diseases of the gastrointestinal tract, IBDs, in particular Crohn's disease (CD) and ulcerative colitis (UC), are those where a strict interplay with HCMV infection has been demonstrated [215]. CD and UC differ in the type of lesions affecting the digestive tract. Indeed, while UC is characterized by constant damage to the rectum and variable and continuous lesions to the colon, CD displays discontinuous lesions of the digestive tract [216]. Activation of IFN-γ-releasing T helper cells (Th1/Th17) and CTL is a common marker of CD, thought to counteract HCMV activity. Conversely, UC is characterized by a Th2/Th9 profile that does not inhibit HCMV replication [217,218]. These key immunological differences may offer some clues as to why HCMV reactivation is an infrequent event during CD flares, whereas it is recurrent in patients affected by UC.

A correlation between HCMV and IBDs was first proposed over 50 years ago [219] on the basis of the observation that treatment of inflamed colonic mucosa with immunosuppressive drugs, such as corticosteroids, favored HCMV reactivation. A role of HCMV in IBD has been very recently corroborated by findings showing that HCMV infection may also complicate UC or CD hospitalizations in terms of increased inpatient mortality, length of stay, and hospital charges [220].

HCMV-induced bowel inflammation follows a general pattern consisting of three phases. The first phase (initiation) involves the release of soluble mediators of inflammation from the mucosa, which serves as a way to recruit latently infected monocytes. In the second phase (reactivation), monocyte activation, and differentiation trigger viral reactivation. In the final phase (consolidation), HCMV starts replicating predominantly in ECs, exacerbating the inflammatory response [221–225]. Although the reported prevalence of HCMV infection in active IBD is highly variable, HCMV infection is regarded by many as an important risk factor for the occurrence and exacerbation of IBD [226]. However, the contribution of HCMV in IBD flare-ups has been recently questioned. While some authors have argued in favor of a significant contribution of the virus in promoting inflammatory flares, others have endorsed a role of HCMV as passive bystander [227–229]. For instance, two cohorts of HCMV-positive and HCMV-negative patients showed similar rates of colectomy, and the specific markers of infection spontaneously disappeared in HCMV-positive patients [230]. In contrast, another group found an association between HCMV infection and enhanced risk of steroid resistance, but no undeniable consensus was actually reached [231,232]. These discrepancies can be to a certain extent reconciled by the fact that the patients enrolled in those studies were affected by different inflammatory diseases (UC or CD), displayed heterogeneous clinical scores, and underwent different treatments. Additionally, inappropriate HCMV detection methods were employed. In-

terestingly, episodes of HCMV-related enterocolitis tend to decrease among IBD patients, suggesting that shifting from a corticosteroid-based maintenance therapy to more effective agents that do not trigger viral reactivation may lessen the risk of HCMV colitis [233].

Additionally, the findings related to HCMV prevalence appear to be highly heterogeneous. For example, a meta-analysis demonstrated that HCMV infection occurred in a percentage of IBD patients ranging from 0.5–100% [234]. Furthermore, an inconsistent percentage of HCMV antigen positivity (10–90%) was reported by three IBD biopsy studies [235–237]. In particular, HCMV tissue infection was observed in 11% of steroid-refractory CD patients vs. 38% of UC patients [238,239]. Moreover, markers of HCMV infection are rarely found in patients with inactive or mild-to-moderate UC [226,240–242], whereas active HCMV infection occurs in 20% to 40% of steroid-refractory UC [243–250], suggesting that HCMV exacerbates inflammation.

The molecular mechanisms underlying the interplay between HCMV and IBD seem to be related to TNF-α, an inflammatory cytokine important for the pathophysiology of IBD. Fittingly, different studies have shown how effective anti-TNFα agents can be in treating IBDs refractory to medical therapy [251,252]. Interestingly, upon binding to the TNF receptor (TNFR), TNF-α promotes NF-κB-mediate transactivation of the IE gene, thereby triggering the differentiation of HCMV latently infected cells and boosting the overall virus growth [253].

The relationship between IBD and HCMV has been studied in more detail using TCR-αKO mice latently infected with MCMV [254,255], a condition thought to replicate HCMV latency. TCR-αKO mice are prone to develop colitis, during which an increase in MCMV replication rates is typically observed. Interestingly, infected cells were identified mostly in the perivascular stroma region (i.e., pericytes) and inflamed colonic mucosa, in good agreement with reports showing that HCMV infection is more pronounced when an inflammatory status coexists [226]. In these sites, neutrophil migration and M1 macrophage presence were detected, further corroborating the notion that HCMV can induce these events in vitro as well [256].

The diagnostic protocol employed to differentiate HCMV-induced colitis from colitis associated with the inflammatory disease itself requires the analysis of viral markers, as clinical or endoscopic symptoms are not sufficient for the differential diagnosis [257–259].

Different methods are now available for the diagnosis of HCMV infection, either indirect (e.g., IgM and IgG detection) or direct ones (e.g., detection of the virus or its components), even though sometimes it is difficult to demonstrate HCMV reactivation from its intestinal reservoir (reviewed in [228,260]). Probably, the most useful method to distinguish refractory from non-refractory IBD is to quantify the HCMV load, since refractory patients display HCMV DNA values higher than 10^3 copies/10^5 cells—either enterocytes or immune cells—in the damaged mucosa [261,262], thus enabling the differentiation of HCMV colitis from mucosal infection.

HCMV infection is a critical issue to be taken into account also when it comes to therapeutic options for IBD patients. Corticosteroids are the first-line therapy for moderate-to-severe IBD flare-ups, but they enhance HCMV reactivation. Another treatment option for UC patients is represented by antivirals. Antiviral therapy is considered the most appropriate approach for moderate-to-severe, steroid-refractory relapse with high viral load values [263]. The main difficulty with applying the appropriate antiviral therapy is the distinction of HCMV reactivation from HCMV colitis as inflammation of the colonic mucosa of UC patients may contribute to reactivating HCMV replication [227,264]. Antiviral treatment allows some patients with steroid-resistant UC and active HCMV infection to avoid colectomy, even though they are poor responder to conventional IBD therapies [265], sometimes restoring the response to immunosuppressive therapies [266]. The response rate with antiviral therapy in patients with steroid-refractory disease showing HCMV reactivation is 72% (range 50–83%) [231,242,244,248]. These data should not be considered as univocal, because most of these patients were simultaneously treated with cyclosporine or granulocytapheresis and antivirals. In addition, those HCMV positive patients who were not treated

with antivirals also showed clinical improvements [230,236,244,267]. Many authors argue that antiviral treatment should be given concomitantly with immunosuppressive therapy to achieve a synergistic effect on both inflammation and viral replication [268,269], especially in the case of anti-TNF-α therapy [228,254]. Finally, an alternative option to treat UC patients with HCMV colitis is represented by the administration of granulocyte/monocyte adsorptive apheresis [244,270] or tacrolimus [230,244,271].

4.4. Metabolic Diseases

Type 1 Diabetes

Type 1 diabetes (T1D) is a chronic disease, characterized by the destruction of pancreatic β-cells, resulting in insulin deficiency. Autoimmune processes triggered by virus infections, combined with genetic susceptibility and environmental factors, have been implicated in the complex pathogenesis of T1D [272,273].

Attempts carried out by different groups to understand if HCMV is involved in the etiology of T1D gave controversial results.

For example, two independent Finnish studies did not establish an association between HCMV and T1D in young children [274,275]. These results confirm a Swedish prospective study about T1D prevalence in congenitally infected infants [276]. Conversely, a strong correlation between positivity for the HCMV genome and autoantibodies against islet cells has been found in PBMCs of Canadian T1D patients [88] as well as in a congenitally HCMV infected child, who developed T1D already at the age of 13 months [277]. Among herpesviruses, also EBV has been suggested to be related to the development of T1D [278]. A more recent paper investigating the relationship between HCMV and EBV with T1D revealed a higher percentage of IgM against HCMV and EBV in T1D patients compared to the control group [279]. These studies collectively suggested that HCMV, and also EBV, could represent a co-factor, rather than a major player, in the development of T1D.

Finally, HCMV is also generally considered an independent risk factor for early developing new-onset posttransplantation diabetes mellitus (PTDM), supported by the observation of its ability to induce the immunological damage of β-cells [280].

5. Conclusions

In recent years, HCMV has gained increasing attention from researchers due to its harmful effects on immunocompromised patients. The tremendous research effort undertaken to understand the mechanisms of HCMV pathogenesis and develop new diagnostic techniques and antiviral drugs has however led to the discovery of novel functions of this virus in other pathophysiological processes such as autoimmunity. In this review, we have summarized past and current literature on the emerging role of HCMV in several ADs, elucidating mechanisms (Figure 2) and related clinical manifestations (Table 1).

Overall, the evidence herein described clearly highlights the widespread ability of HCMV to manipulate the immune system, which may lead to self-tolerance breakdown in genetically predisposed individuals. Many hypotheses support that HCMV infection have a role in ADs. HCMV display a high seroprevalence in adults; in the USA, Europe and Australia, HCMV seroprevalence is variable, ranging between 36% and 77%, while in developing countries and in particular sub-Saharan Africa, HCMV is highly endemic with a seropositivity rate up to 100% [281]. A strengthening explanation for the high incidence of HCMV in AD patients in developing countries could be related to the high prevalence of ADs in the general population and the endemic state of HCMV with a rate approaching 100% in some areas [281].

Primary and secondary HCMV infections seem to be highly effective in shifting the balance toward immune dysregulation, which eventually triggers the initiation or perpetuation of ADs. There are also a few studies claiming a protective role of HCMV in ADs, such as in the case of MS [98], which may be easily explained by the fact that HCMV during the course of evolution has devised a number of strategies that limit inflammation and tissue damage of the host to preserve virus–host coexistence [282].

Overall, the development of new diagnostic markers to detect the presence of HCMV in AD patients may help clinicians better predict the type of clinical manifestations and the extent of disease progression. Furthermore, it is envisaged that the adoption of antivirals against HCMV in combination with immunosuppressive therapy may represent a viable therapeutic solution for certain ADs.

As large epidemiological studies are clearly needed to draw any definitive conclusions on the role of HCMV in AD pathogenesis, the availability of effective HCMV vaccines, currently in clinical development, could not only unravel the impact of HCMV on Ads, but also improve the quality of life of AD patients.

Table 1. Autoimmune diseases which have been triggered by or associated with HCMV.

Autoimmune Diseases	References *
Rheumatologic diseases	
Systemic Lupus Erythematosus	[86,87,89,118–124,126,128–134]
Systemic sclerosis	[33,83,111,138–141,143–147,149]
Rheumatoid arthritis	[92,95,100,144,151–156,179,183,185]
Neurological diseases	
Multiple sclerosis	[98,200–205,209]
Enteropathies	
Crohn disease & ulcerative colitis	[217–256,263–271]
Metabolic diseases	
Type 1 diabetes	[88,274–280]

* References cite case reports, studies or aspects of pathogenesis in each case.

Author Contributions: M.B., F.G. and S.P. developed the ideas and drafted the manuscript. V.D., M.B., F.G., G.G., S.S., S.P. and G.R. wrote sections of the manuscript. C.A. and S.F.C.P. drew the figures. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Italian Ministry of Education, University and Research-MIUR (PRIN 20178ALPCM) to V.D., "Cassa di Risparmio" Foundation of Turin, Italy (RF = 2019.2273) to V.D., University of Turin, Italy ("Ricerca Locale" 2020) to V.D., M.B., F.G., S.S.

Acknowledgments: We thank Marcello Arsura for editing and proofreading the manuscript.

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

References

1. Wang, L.; Wang, F.-S.; Gershwin, M.E. Human Autoimmune Diseases: A Comprehensive Update. *J. Intern. Med.* **2015**, *278*, 369–395. [CrossRef]
2. Selmi, C.; Mayo, M.J.; Bach, N.; Ishibashi, H.; Invernizzi, P.; Gish, R.G.; Gordon, S.C.; Wright, H.I.; Zweiban, B.; Podda, M.; et al. Primary Biliary Cirrhosis in Monozygotic and Dizygotic Twins: Genetics, Epigenetics, and Environment. *Gastroenterology* **2004**, *127*, 485–492. [CrossRef] [PubMed]
3. Perricone, C.; Versini, M.; Ben-Ami, D.; Gertel, S.; Watad, A.; Segel, M.J.; Ceccarelli, F.; Conti, F.; Cantarini, L.; Bogdanos, D.P.; et al. Smoke and Autoimmunity: The Fire behind the Disease. *Autoimmun. Rev.* **2016**, *15*, 354–374. [CrossRef] [PubMed]
4. Lammert, C. Genetic and Environmental Risk Factors for Autoimmune Hepatitis. *Clin. Liver Dis.* **2019**, *14*, 29–32. [CrossRef] [PubMed]
5. Deitiker, P.; Atassi, M.Z. Non-MHC Genes Linked to Autoimmune Disease. *Crit. Rev. Immunol.* **2012**, *32*, 193–285. [CrossRef]
6. Hu, X.; Daly, M. What Have We Learned from Six Years of GWAS in Autoimmune Diseases, and What Is Next? *Curr. Opin. Immunol.* **2012**, *24*, 571–575. [CrossRef]
7. Fairweather, D.; Rose, N.R. Women and Autoimmune Diseases. *Emerg. Infect. Dis.* **2004**, *10*, 2005–2011. [CrossRef]
8. Stern-Ginossar, N.; Weisburd, B.; Michalski, A.; Le, V.T.K.; Hein, M.Y.; Huang, S.-X.; Ma, M.; Shen, B.; Qian, S.-B.; Hengel, H.; et al. Decoding Human Cytomegalovirus. *Science* **2012**, *338*, 1088–1093. [CrossRef]
9. Shnayder, M.; Nachshon, A.; Krishna, B.; Poole, E.; Boshkov, A.; Binyamin, A.; Maza, I.; Sinclair, J.; Schwartz, M.; Stern-Ginossar, N. Defining the Transcriptional Landscape during Cytomegalovirus Latency with Single-Cell RNA Sequencing. *mBio* **2018**, *9*. [CrossRef]
10. DiNardo, A.R.; Netea, M.G.; Musher, D.M. Postinfectious Epigenetic Immune Modifications—A Double-Edged Sword. *N. Engl. J. Med.* **2021**, *384*, 261–270. [CrossRef] [PubMed]

11. Liu, X.; Hummel, M.; Abecassis, M. Epigenetic Regulation of Cellular and Cytomegalovirus Genes during Myeloid Cell Development. *Intern. Med. Rev.* **2017**, *3*. [CrossRef]
12. Kananen, L.; Nevalainen, T.; Jylhävä, J.; Marttila, S.; Hervonen, A.; Jylhä, M.; Hurme, M. Cytomegalovirus Infection Accelerates Epigenetic Aging. *Exp. Gerontol.* **2015**, *72*, 227–229. [CrossRef] [PubMed]
13. Surace, A.E.A.; Hedrich, C.M. The Role of Epigenetics in Autoimmune/Inflammatory Disease. *Front. Immunol.* **2019**, *10*, 1525. [CrossRef]
14. Dunn, W.; Chou, C.; Li, H.; Hai, R.; Patterson, D.; Stolc, V.; Zhu, H.; Liu, F. Functional Profiling of a Human Cytomegalovirus Genome. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 14223–14228. [CrossRef]
15. McSharry, B.P.; Avdic, S.; Slobedman, B. Human Cytomegalovirus Encoded Homologs of Cytokines, Chemokines and Their Receptors: Roles in Immunomodulation. *Viruses* **2012**, *4*, 2448–2470. [CrossRef]
16. Halenius, A.; Hengel, H. Human Cytomegalovirus and Autoimmune Disease. *Biomed. Res. Int.* **2014**, *2014*, 472978. [CrossRef]
17. Galitska, G.; Biolatti, M.; De Andrea, M.; Leone, A.; Coscia, A.; Bertolotti, L.; Ala, U.; Bertino, E.; Dell'Oste, V.; Landolfo, S. Biological Relevance of Cytomegalovirus Genetic Variability in Congenitally and Postnatally Infected Children. *J. Clin. Virol.* **2018**, *108*, 132–140. [CrossRef] [PubMed]
18. Gugliesi, F.; Coscia, A.; Griffante, G.; Galitska, G.; Pasquero, S.; Albano, C.; Biolatti, M. Where Do We Stand after Decades of Studying Human Cytomegalovirus? *Microorganisms* **2020**, *8*, 685. [CrossRef]
19. Berry, R.; Watson, G.M.; Jonjic, S.; Degli-Esposti, M.A.; Rossjohn, J. Modulation of Innate and Adaptive Immunity by Cytomegaloviruses. *Nat. Rev. Immunol.* **2020**, *20*, 113–127. [CrossRef]
20. Sinzger, C.; Schmidt, K.; Knapp, J.; Kahl, M.; Beck, R.; Waldman, J.; Hebart, H.; Einsele, H.; Jahn, G. Modification of Human Cytomegalovirus Tropism through Propagation in Vitro Is Associated with Changes in the Viral Genome. *J. Gen. Virol.* **1999**, *80*, 2867–2877. [CrossRef] [PubMed]
21. Sinzger, C.; Digel, M.; Jahn, G. Cytomegalovirus Cell Tropism. *Curr. Top. Microbiol. Immunol.* **2008**, *325*, 63–83.
22. Nguyen, C.C.; Kamil, J.P. Pathogen at the Gates: Human Cytomegalovirus Entry and Cell Tropism. *Viruses* **2018**, *10*, 704. [CrossRef]
23. Gerna, G.; Kabanova, A.; Lilleri, D. Human Cytomegalovirus Cell Tropism and Host Cell Receptors. *Vaccines* **2019**, *7*, 70. [CrossRef] [PubMed]
24. Zuhair, M.; Smit, G.S.A.; Wallis, G.; Jabbar, F.; Smith, C.; Devleesschauwer, B.; Griffiths, P. Estimation of the Worldwide Seroprevalence of Cytomegalovirus: A Systematic Review and Meta-Analysis. *Rev. Med. Virol.* **2019**, *29*, e2034. [CrossRef] [PubMed]
25. HoHsieh, A.; Wang, C.M.; Wu, Y.-J.J.; Chen, A.; Chang, M.-I.; Chen, J.-Y. B Cell Epitope of Human Cytomegalovirus Phosphoprotein 65 (HCMV Pp65) Induced Anti-DsDNA Antibody in BALB/c Mice. *Arthritis Res.* **2017**, *19*, 65. [CrossRef] [PubMed]
26. Varani, S.; Mastroianni, A.; Frascaroli, G.; Tammik, C.; Rahbar, A.; Christensson, M.; Rossini, G.; Landini, M.P.; Söderberg-Nauclér, C. Generalized Wegener's Granulomatosis in an Immunocompetent Adult after Cytomegalovirus Mononucleosis and Bacterial Urinary Tract Infection. *Arthritis Rheum.* **2009**, *60*, 1558–1562. [CrossRef]
27. Giugni, T.D.; Söderberg, C.; Ham, D.J.; Bautista, R.M.; Hedlund, K.O.; Möller, E.; Zaia, J.A. Neutralization of Human Cytomegalovirus by Human CD13-Specific Antibodies. *J. Infect. Dis.* **1996**, *173*, 1062–1071. [CrossRef] [PubMed]
28. Soderberg, C.; Sumitran-Karuppan, S.; Ljungman, P.; Moller, E. CD13-Specific Autoimmunity in Cytomegalovirus-Infected Immunocompromised Patients. *Transplantation* **1996**, *61*, 594–600. [CrossRef] [PubMed]
29. Mengarelli, A.; Minotti, C.; Palumbo, G.; Arcieri, P.; Gentile, G.; Iori, A.P.; Arcese, W.; Mandelli, F.; Avvisati, G. High Levels of Antiphospholipid Antibodies Are Associated with Cytomegalovirus Infection in Unrelated Bone Marrow and Cord Blood Allogeneic Stem Cell Transplantation. *Br. J. Haematol.* **2000**, *108*, 126–131. [CrossRef]
30. Toyoda, M.; Galfayan, K.; Galera, O.A.; Petrosian, A.; Czer, L.S.; Jordan, S.C. Cytomegalovirus Infection Induces Anti-Endothelial Cell Antibodies in Cardiac and Renal Allograft Recipients. *Transplant. Immunol.* **1997**, *5*, 104–111. [CrossRef]
31. Wager, O.; Räsänen, J.A.; Hagman, A.; Klemola, E. Mixed Cryoimmunoglobulinaemia in Infectious Mononucleois and Cytomegalovirus Mononucleosis. *Int. Arch. Allergy Appl. Immunol.* **1968**, *34*, 345–361. [CrossRef]
32. Kantor, G.L.; Goldberg, L.S.; Johnson, B.L.; Derechin, M.M.; Barnett, E.V. Immunologic Abnormalities Induced by Postperfusion Cytomegalovirus Infection. *Ann. Intern. Med.* **1970**, *73*, 553–558. [CrossRef] [PubMed]
33. Lunardi, C.; Dolcino, M.; Peterlana, D.; Bason, C.; Navone, R.; Tamassia, N.; Beri, R.; Corrocher, R.; Puccetti, A. Antibodies against Human Cytomegalovirus in the Pathogenesis of Systemic Sclerosis: A Gene Array Approach. *PLoS Med.* **2006**, *3*, e2. [CrossRef] [PubMed]
34. Streblow, D.N.; Orloff, S.L.; Nelson, J.A. Acceleration of Allograft Failure by Cytomegalovirus. *Curr. Opin. Immunol.* **2007**, *19*, 577–582. [CrossRef]
35. Amaya-Amaya, J.; Montoya-Sánchez, L.; Rojas-Villarraga, A. Cardiovascular Involvement in Autoimmune Diseases. *Biomed. Res. Int.* **2014**, *2014*, 367359. [CrossRef] [PubMed]
36. van de Berg, P.J.; Heutinck, K.M.; Raabe, R.; Minnee, R.C.; Young, S.L.; van Donselaar-van der Pant, K.A.; Bemelman, F.J.; van Lier, R.A.; ten Berge, I.J. Human Cytomegalovirus Induces Systemic Immune Activation Characterized by a Type 1 Cytokine Signature. *J. Infect. Dis.* **2010**, *202*, 690–699. [CrossRef]

37. George, M.J.; Snydman, D.R.; Werner, B.G.; Griffith, J.; Falagas, M.E.; Dougherty, N.N.; Rubin, R.H. The Independent Role of Cytomegalovirus as a Risk Factor for Invasive Fungal Disease in Orthotopic Liver Transplant Recipients. Boston Center for Liver Transplantation CMVIG-Study Group. Cytogam, MedImmune, Inc. Gaithersburg, Maryland. *Am. J. Med.* **1997**, *103*, 106–113. [CrossRef]
38. Forte, E.; Zhang, Z.; Thorp, E.B.; Hummel, M. Cytomegalovirus Latency and Reactivation: An Intricate Interplay With the Host Immune Response. *Front. Cell Infect. Microbiol.* **2020**, *10*, 130. [CrossRef]
39. Dell'Oste, V.; Biolatti, M.; Galitska, G.; Griffante, G.; Gugliesi, F.; Pasquero, S.; Zingoni, A.; Cerboni, C.; De Andrea, M. Tuning the Orchestra: HCMV vs. Innate Immunity. *Front. Microbiol.* **2020**, *11*, 661. [CrossRef]
40. Ye, L.; Qian, Y.; Yu, W.; Guo, G.; Wang, H.; Xue, X. Functional Profile of Human Cytomegalovirus Genes and Their Associated Diseases: A Review. *Front. Microbiol.* **2020**, *11*, 2104. [CrossRef]
41. Manandhar, T.; Hò, G.-G.T.; Pump, W.C.; Blasczyk, R.; Bade-Doeding, C. Battle between Host Immune Cellular Responses and HCMV Immune Evasion. *Int. J. Mol. Sci.* **2019**, *20*, 3626. [CrossRef]
42. Hewitt, E.W. The MHC Class I Antigen Presentation Pathway: Strategies for Viral Immune Evasion. *Immunology* **2003**, *110*, 163–169. [CrossRef] [PubMed]
43. Kim, S.; Lee, S.; Shin, J.; Kim, Y.; Evnouchidou, I.; Kim, D.; Kim, Y.-K.; Kim, Y.-E.; Ahn, J.-H.; Riddell, S.R.; et al. Human Cytomegalovirus MicroRNA MiR-US4-1 Inhibits CD8(+) T Cell Responses by Targeting the Aminopeptidase ERAP1. *Nat. Immunol.* **2011**, *12*, 984–991. [CrossRef]
44. Romania, P.; Cifaldi, L.; Pignoloni, B.; Starc, N.; D'Alicandro, V.; Melaiu, O.; Li Pira, G.; Giorda, E.; Carrozzo, R.; Bergvall, M.; et al. Identification of a Genetic Variation in ERAP1 Aminopeptidase That Prevents Human Cytomegalovirus MiR-UL112-5p-Mediated Immunoevasion. *Cell Rep.* **2017**, *20*, 846–853. [CrossRef]
45. Pérez-Carmona, N.; Martínez-Vicente, P.; Farré, D.; Gabaev, I.; Messerle, M.; Engel, P.; Angulo, A. A Prominent Role of the Human Cytomegalovirus UL8 Glycoprotein in Restraining Proinflammatory Cytokine Production by Myeloid Cells at Late Times during Infection. *J. Virol.* **2018**, *92*. [CrossRef]
46. Bruno, L.; Cortese, M.; Monda, G.; Gentile, M.; Calò, S.; Schiavetti, F.; Zedda, L.; Cattaneo, E.; Piccioli, D.; Schaefer, M.; et al. Human Cytomegalovirus PUL10 Interacts with Leukocytes and Impairs TCR-Mediated T-Cell Activation. *Immunol Cell Biol.* **2016**, *94*, 849–860. [CrossRef] [PubMed]
47. Akira, S.; Uematsu, S.; Takeuchi, O. Pathogen Recognition and Innate Immunity. *Cell* **2006**, *124*, 783–801. [CrossRef]
48. Galitska, G.; Biolatti, M.; Griffante, G.; Gugliesi, F.; Pasquero, S.; Dell'Oste, V.; Landolfo, S. Catch Me If You Can: The Arms Race between Human Cytomegalovirus and the Innate Immune System. *Future Virol.* **2019**, *14*, 247–263. [CrossRef]
49. Biolatti, M.; Gugliesi, F.; Dell'Oste, V.; Landolfo, S. Modulation of the Innate Immune Response by Human Cytomegalovirus. *Infect. Genet. Evol.* **2018**, *64*, 105–114. [CrossRef] [PubMed]
50. Biolatti, M.; Dell'Oste, V.; Pautasso, S.; Gugliesi, F.; von Einem, J.; Krapp, C.; Jakobsen, M.R.; Borgogna, C.; Gariglio, M.; De Andrea, M.; et al. Human Cytomegalovirus Tegument Protein Pp65 (PUL83) Dampens Type I Interferon Production by Inactivating the DNA Sensor CGAS without Affecting STING. *J. Virol.* **2018**, *92*. [CrossRef] [PubMed]
51. Huang, Z.-F.; Zou, H.-M.; Liao, B.-W.; Zhang, H.-Y.; Yang, Y.; Fu, Y.-Z.; Wang, S.-Y.; Luo, M.-H.; Wang, Y.-Y. Human Cytomegalovirus Protein UL31 Inhibits DNA Sensing of CGAS to Mediate Immune Evasion. *Cell Host Microbe* **2018**, *24*, 69–80.e4. [CrossRef] [PubMed]
52. Fu, Y.-Z.; Su, S.; Gao, Y.-Q.; Wang, P.-P.; Huang, Z.-F.; Hu, M.-M.; Luo, W.-W.; Li, S.; Luo, M.-H.; Wang, Y.-Y.; et al. Human Cytomegalovirus Tegument Protein UL82 Inhibits STING-Mediated Signaling to Evade Antiviral Immunity. *Cell Host Microbe* **2017**, *21*, 231–243. [CrossRef] [PubMed]
53. Fu, Y.-Z.; Guo, Y.; Zou, H.-M.; Su, S.; Wang, S.-Y.; Yang, Q.; Luo, M.-H.; Wang, Y.-Y. Human Cytomegalovirus Protein UL42 Antagonizes CGAS/MITA-Mediated Innate Antiviral Response. *PLoS Pathog.* **2019**, *15*, e1007691. [CrossRef] [PubMed]
54. Choi, H.J.; Park, A.; Kang, S.; Lee, E.; Lee, T.A.; Ra, E.A.; Lee, J.; Lee, S.; Park, B. Human Cytomegalovirus-Encoded US9 Targets MAVS and STING Signaling to Evade Type I Interferon Immune Responses. *Nat. Commun.* **2018**, *9*, 125. [CrossRef] [PubMed]
55. Taylor, R.T.; Bresnahan, W.A. Human Cytomegalovirus IE86 Attenuates Virus- and Tumor Necrosis Factor Alpha-Induced NFkappaB-Dependent Gene Expression. *J. Virol.* **2006**, *80*, 10763–10771. [CrossRef] [PubMed]
56. Kim, J.-E.; Kim, Y.-E.; Stinski, M.F.; Ahn, J.-H.; Song, Y.-J. Human Cytomegalovirus IE2 86 KDa Protein Induces STING Degradation and Inhibits CGAMP-Mediated IFN-β Induction. *Front. Microbiol.* **2017**, *8*, 1854. [CrossRef]
57. Zingoni, A.; Molfetta, R.; Fionda, C.; Soriani, A.; Paolini, R.; Cippitelli, M.; Cerboni, C.; Santoni, A. NKG2D and Its Ligands: "One for All, All for One". *Front. Immunol.* **2018**, *9*, 476. [CrossRef] [PubMed]
58. Lam, V.C.; Lanier, L.L. NK Cells in Host Responses to Viral Infections. *Curr. Opin. Immunol.* **2017**, *44*, 43–51. [CrossRef] [PubMed]
59. Patel, M.; Vlahava, V.-M.; Forbes, S.K.; Fielding, C.A.; Stanton, R.J.; Wang, E.C.Y. HCMV-Encoded NK Modulators: Lessons From in Vitro and in Vivo Genetic Variation. *Front. Immunol.* **2018**, *9*, 2214. [CrossRef]
60. Schmiedel, D.; Mandelboim, O. Disarming Cellular Alarm Systems-Manipulation of Stress-Induced NKG2D Ligands by Human Herpesviruses. *Front. Immunol.* **2017**, *8*, 390. [CrossRef]
61. Cosman, D.; Müllberg, J.; Sutherland, C.L.; Chin, W.; Armitage, R.; Fanslow, W.; Kubin, M.; Chalupny, N.J. ULBPs, Novel MHC Class I-Related Molecules, Bind to CMV Glycoprotein UL16 and Stimulate NK Cytotoxicity through the NKG2D Receptor. *Immunity* **2001**, *14*, 123–133. [CrossRef]

62. Kubin, M.; Cassiano, L.; Chalupny, J.; Chin, W.; Cosman, D.; Fanslow, W.; Müllberg, J.; Rousseau, A.M.; Ulrich, D.; Armitage, R. ULBP1, 2, 3: Novel MHC Class I-Related Molecules That Bind to Human Cytomegalovirus Glycoprotein UL16, Activate NK Cells. *Eur. J. Immunol.* **2001**, *31*, 1428–1437. [CrossRef]
63. Rölle, A.; Mousavi-Jazi, M.; Eriksson, M.; Odeberg, J.; Söderberg-Nauclér, C.; Cosman, D.; Kärre, K.; Cerboni, C. Effects of Human Cytomegalovirus Infection on Ligands for the Activating NKG2D Receptor of NK Cells: Up-Regulation of UL16-Binding Protein (ULBP)1 and ULBP2 Is Counteracted by the Viral UL16 Protein. *J. Immunol.* **2003**, *171*, 902–908. [CrossRef]
64. Eagle, R.A.; Traherne, J.A.; Hair, J.R.; Jafferji, I.; Trowsdale, J. ULBP6/RAET1L Is an Additional Human NKG2D Ligand. *Eur. J. Immunol.* **2009**, *39*, 3207–3216. [CrossRef] [PubMed]
65. Ashiru, O.; Bennett, N.J.; Boyle, L.H.; Thomas, M.; Trowsdale, J.; Wills, M.R. NKG2D Ligand MICA Is Retained in the Cis-Golgi Apparatus by Human Cytomegalovirus Protein UL142. *J. Virol.* **2009**, *83*, 12345–12354. [CrossRef]
66. Bennett, N.J.; Ashiru, O.; Morgan, F.J.E.; Pang, Y.; Okecha, G.; Eagle, R.A.; Trowsdale, J.; Sissons, J.G.P.; Wills, M.R. Intracellular Sequestration of the NKG2D Ligand ULBP3 by Human Cytomegalovirus. *J. Immunol.* **2010**, *185*, 1093–1102. [CrossRef]
67. Fielding, C.A.; Weekes, M.P.; Nobre, L.V.; Ruckova, E.; Wilkie, G.S.; Paulo, J.A.; Chang, C.; Suárez, N.M.; Davies, J.A.; Antrobus, R.; et al. Control of Immune Ligands by Members of a Cytomegalovirus Gene Expansion Suppresses Natural Killer Cell Activation. *Elife* **2017**, *6*. [CrossRef]
68. Charpak-Amikam, Y.; Kubsch, T.; Seidel, E.; Oiknine-Djian, E.; Cavaletto, N.; Yamin, R.; Schmiedel, D.; Wolf, D.; Gribaudo, G.; Messerle, M.; et al. Human Cytomegalovirus Escapes Immune Recognition by NK Cells through the Downregulation of B7-H6 by the Viral Genes US18 and US20. *Sci. Rep.* **2017**, *7*, 8661. [CrossRef]
69. Jenks, J.A.; Goodwin, M.L.; Permar, S.R. The Roles of Host and Viral Antibody Fc Receptors in Herpes Simplex Virus (HSV) and Human Cytomegalovirus (HCMV) Infections and Immunity. *Front. Immunol.* **2019**, *10*. [CrossRef]
70. Atalay, R.; Zimmermann, A.; Wagner, M.; Borst, E.; Benz, C.; Messerle, M.; Hengel, H. Identification and Expression of Human Cytomegalovirus Transcription Units Coding for Two Distinct Fcγ Receptor Homologs. *J. Virol.* **2002**, *76*, 8596–8608. [CrossRef]
71. Corrales-Aguilar, E.; Trilling, M.; Hunold, K.; Fiedler, M.; Le, V.T.K.; Reinhard, H.; Ehrhardt, K.; Mercé-Maldonado, E.; Aliyev, E.; Zimmermann, A.; et al. Human Cytomegalovirus Fcγ Binding Proteins Gp34 and Gp68 Antagonize Fcγ Receptors I, II and III. *PLoS Pathog.* **2014**, *10*, e1004131. [CrossRef]
72. Cortese, M.; Calò, S.; D'Aurizio, R.; Lilja, A.; Pacchiani, N.; Merola, M. Recombinant Human Cytomegalovirus (HCMV) RL13 Binds Human Immunoglobulin G Fc. *PLoS ONE* **2012**, *7*, e50166. [CrossRef]
73. Sprague, E.R.; Reinhard, H.; Cheung, E.J.; Farley, A.H.; Trujillo, R.D.; Hengel, H.; Bjorkman, P.J. The Human Cytomegalovirus Fc Receptor Gp68 Binds the Fc CH2-CH3 Interface of Immunoglobulin G. *J. Virol.* **2008**, *82*, 3490–3499. [CrossRef]
74. Perrier, S.; Serre, A.F.; Dubost, J.J.; Beaujon, G.; Plazonnet, M.P.; Albuisson, E.; Sauvezie, B. Increased Serum Levels of Interleukin 10 in Sjögren's Syndrome; Correlation with Increased IgG1. *J. Rheumatol.* **2000**, *27*, 935–939.
75. Elazeem, M.I.A.; Mohammed, R.A.; Abdallah, N.H. Correlation of Serum Interleukin-10 Level with Disease Activity and Severity in Systemic Lupus Erythematosus. *Egypt Rheumatol. Rehabil.* **2018**, *45*, 25–33. [CrossRef]
76. Manolova, I.; Ivanova, M.; Stanilova, S. Gene Polymorphisms of Immunoregulatory Cytokines IL-10 and TGF-B1 in Systemic Lupus Erythematosus. *Genes Autoimmun. Intracell. Signal. Microbiome Contrib.* **2013**. [CrossRef]
77. Colafrancesco, S.; Ciccacci, C.; Priori, R.; Latini, A.; Picarelli, G.; Arienzo, F.; Novelli, G.; Valesini, G.; Perricone, C.; Borgiani, P. STAT4, TRAF3IP2, IL10, and HCP5 Polymorphisms in Sjögren's Syndrome: Association with Disease Susceptibility and Clinical Aspects. Available online: https://www.hindawi.com/journals/jir/2019/7682827/ (accessed on 2 February 2021).
78. Ciccacci, C.; Perricone, C.; Ceccarelli, F.; Rufini, S.; Fusco, D.D.; Alessandri, C.; Spinelli, F.R.; Cipriano, E.; Novelli, G.; Valesini, G.; et al. A Multilocus Genetic Study in a Cohort of Italian SLE Patients Confirms the Association with STAT4 Gene and Describes a New Association with HCP5 Gene. *PLoS ONE* **2014**, *9*, e111991. [CrossRef] [PubMed]
79. Ji, J.D.; Lee, W.J.; Kong, K.A.; Woo, J.H.; Choi, S.J.; Lee, Y.H.; Song, G.G. Association of STAT4 Polymorphism with Rheumatoid Arthritis and Systemic Lupus Erythematosus: A Meta-Analysis. *Mol. Biol. Rep.* **2010**, *37*, 141–147. [CrossRef] [PubMed]
80. Hagberg, N.; Rönnblom, L. Interferon-α Enhances the IL-12-Induced STAT4 Activation Selectively in Carriers of the STAT4 SLE Risk Allele Rs7574865[T]. *Ann. Rheum. Dis.* **2019**, *78*, 429–431. [CrossRef]
81. Nagata, K.; Hayashi, K. Epstein-Barr Virus Reactivation-Induced Immunoglobulin Production: Significance on Autoimmunity. *Microorganisms* **2020**, *8*, 1875. [CrossRef]
82. Muryoi, T.; Kasturi, K.N.; Kafina, M.J.; Cram, D.S.; Harrison, L.C.; Sasaki, T.; Bona, C.A. Antitopoisomerase I Monoclonal Autoantibodies from Scleroderma Patients and Tight Skin Mouse Interact with Similar Epitopes. *J. Exp. Med.* **1992**, *175*, 1103–1109. [CrossRef]
83. Lunardi, C.; Bason, C.; Navone, R.; Millo, E.; Damonte, G.; Corrocher, R.; Puccetti, A. Systemic Sclerosis Immunoglobulin G Autoantibodies Bind the Human Cytomegalovirus Late Protein UL94 and Induce Apoptosis in Human Endothelial Cells. *Nat. Med.* **2000**, *6*, 1183–1186. [CrossRef] [PubMed]
84. Chang, M.; Pan, M.-R.; Chen, D.-Y.; Lan, J.-L. Human Cytomegalovirus Pp65 Lower Matrix Protein: A Humoral Immunogen for Systemic Lupus Erythematosus Patients and Autoantibody Accelerator for NZB/W F1 Mice. *Clin. Exp. Immunol.* **2006**, *143*, 167–179. [CrossRef] [PubMed]
85. Liu, Y.; Mu, R.; Gao, Y.-P.; Dong, J.; Zhu, L.; Ma, Y.; Li, Y.-H.; Zhang, H.-Q.; Han, D.; Zhang, Y.; et al. A Cytomegalovirus Peptide-Specific Antibody Alters Natural Killer Cell Homeostasis and Is Shared in Several Autoimmune Diseases. *Cell Host Microbe* **2016**, *19*, 400–408. [CrossRef]

86. Hsieh, A.-H.; Jhou, Y.-J.; Liang, C.-T.; Chang, M.; Wang, S.-L. Fragment of Tegument Protein Pp65 of Human Cytomegalovirus Induces Autoantibodies in BALB/c Mice. *Arthritis Res.* **2011**, *13*, R162. [CrossRef] [PubMed]
87. Neo, J.Y.J.; Wee, S.Y.K.; Bonne, I.; Tay, S.H.; Raida, M.; Jovanovic, V.; Fairhurst, A.-M.; Lu, J.; Hanson, B.J.; MacAry, P.A. Characterisation of a Human Antibody That Potentially Links Cytomegalovirus Infection with Systemic Lupus Erythematosus. *Sci. Rep.* **2019**, *9*, 9998. [CrossRef]
88. Pak, C.Y.; Cha, C.Y.; Rajotte, R.V.; McArthur, R.G.; Yoon, J.W. Human Pancreatic Islet Cell Specific 38 Kilodalton Autoantigen Identified by Cytomegalovirus-Induced Monoclonal Islet Cell Autoantibody. *Diabetologia* **1990**, *33*, 569–572. [CrossRef]
89. Curtis, H.A.; Singh, T.; Newkirk, M.M. Recombinant Cytomegalovirus Glycoprotein GB (UL55) Induces an Autoantibody Response to the U1-70 KDa Small Nuclear Ribonucleoprotein. *Eur. J. Immunol.* **1999**, *29*, 3643–3653. [CrossRef]
90. Varani, S.; Cederarv, M.; Feld, S.; Tammik, C.; Frascaroli, G.; Landini, M.P.; Söderberg-Nauclér, C. Human Cytomegalovirus Differentially Controls B Cell and T Cell Responses through Effects on Plasmacytoid Dendritic Cells. *J. Immunol.* **2007**, *179*, 7767–7776. [CrossRef]
91. Xu, H.; Dong, P.; Ma, X.; Song, D.; Xue, D.; Xu, R.; Lu, H.; He, X. B Cell-Activating Factor Regulates the Survival of B Lymphocytes Infected with Human Cytomegalovirus. *Immunol. Lett.* **2017**, *187*, 1–6. [CrossRef]
92. van Leeuwen, E.M.M.; Remmerswaal, E.B.M.; Vossen, M.T.M.; Rowshani, A.T.; Wertheim-van Dillen, P.M.E.; van Lier, R.A.W.; ten Berge, I.J.M. Emergence of a CD4+CD28- Granzyme B+, Cytomegalovirus-Specific T Cell Subset after Recovery of Primary Cytomegalovirus Infection. *J. Immunol.* **2004**, *173*, 1834–1841. [CrossRef]
93. Fasth, A.E.R.; Dastmalchi, M.; Rahbar, A.; Salomonsson, S.; Pandya, J.M.; Lindroos, E.; Nennesmo, I.; Malmberg, K.-J.; Söderberg-Nauclér, C.; Trollmo, C.; et al. T Cell Infiltrates in the Muscles of Patients with Dermatomyositis and Polymyositis Are Dominated by CD28null T Cells. *J. Immunol.* **2009**, *183*, 4792–4799. [CrossRef] [PubMed]
94. Gerli, R.; Schillaci, G.; Giordano, A.; Bocci, E.B.; Bistoni, O.; Vaudo, G.; Marchesi, S.; Pirro, M.; Ragni, F.; Shoenfeld, Y.; et al. CD4+CD28− T Lymphocytes Contribute to Early Atherosclerotic Damage in Rheumatoid Arthritis Patients. *Circulation* **2004**, *109*, 2744–2748. [CrossRef]
95. Bano, A.; Pera, A.; Almoukayed, A.; Clarke, T.H.S.; Kirmani, S.; Davies, K.A.; Kern, F. CD28null CD4 T-Cell Expansions in Autoimmune Disease Suggest a Link with Cytomegalovirus Infection. *F1000Res* **2019**, *8*, 327. [CrossRef]
96. Chanouzas, D.; Sagmeister, M.; Faustini, S.; Nightingale, P.; Richter, A.; Ferro, C.J.; Morgan, M.D.; Moss, P.; Harper, L. Subclinical Reactivation of Cytomegalovirus Drives CD4+CD28null T-Cell Expansion and Impaired Immune Response to Pneumococcal Vaccination in Antineutrophil Cytoplasmic Antibody-Associated Vasculitis. *J. Infect. Dis.* **2019**, *219*, 234–244. [CrossRef]
97. Wu, C.-S.; Chyuan, I.-T.; Chiu, Y.-L.; Chen, W.-L.; Shen, C.-Y.; Hsu, P.-N. Preserved Specific Anti-Viral T-Cell Response but Associated with Decreased Lupus Activity in SLE Patients with Cytomegalovirus Infection. *Rheumatology* **2020**, *59*, 3340–3349. [CrossRef] [PubMed]
98. Zabalza, A.; Vera, A.; Alari-Pahissa, E.; Munteis, E.; Moreira, A.; Yélamos, J.; Llop, M.; López-Botet, M.; Martínez-Rodríguez, J.E. Impact of Cytomegalovirus Infection on B Cell Differentiation and Cytokine Production in Multiple Sclerosis. *J. Neuroinflammation* **2020**, *17*, 161. [CrossRef]
99. Almanzar, G.; Schmalzing, M.; Trippen, R.; Höfner, K.; Weißbrich, B.; Geissinger, E.; Meyer, T.; Liese, J.; Tony, H.-P.; Prelog, M. Significant IFNγ Responses of CD8+ T Cells in CMV-Seropositive Individuals with Autoimmune Arthritis. *J. Clin. Virol.* **2016**, *77*, 77–84. [CrossRef]
100. Rothe, K.; Quandt, D.; Schubert, K.; Rossol, M.; Klingner, M.; Jasinski-Bergner, S.; Scholz, R.; Seliger, B.; Pierer, M.; Baerwald, C.; et al. Latent Cytomegalovirus Infection in Rheumatoid Arthritis and Increased Frequencies of Cytolytic LIR-1+CD8+ T Cells. *Arthritis Rheumatol.* **2016**, *68*, 337–346. [CrossRef] [PubMed]
101. Janahi, E.M.A.; Das, S.; Bhattacharya, S.N.; Haque, S.; Akhter, N.; Jawed, A.; Wahid, M.; Mandal, R.K.; Lohani, M.; Areeshi, M.Y.; et al. Cytomegalovirus Aggravates the Autoimmune Phenomenon in Systemic Autoimmune Diseases. *Microb. Pathog.* **2018**, *120*, 132–139. [CrossRef]
102. Arcangeletti, M.-C.; Maccari, C.; Vescovini, R.; Volpi, R.; Giuggioli, D.; Sighinolfi, G.; De Conto, F.; Chezzi, C.; Calderaro, A.; Ferri, C. A Paradigmatic Interplay between Human Cytomegalovirus and Host Immune System: Possible Involvement of Viral Antigen-Driven CD8+ T Cell Responses in Systemic Sclerosis. *Viruses* **2018**, *10*, 508. [CrossRef]
103. Arcangeletti, M.-C.; D'Accolti, M.; Maccari, C.; Soffritti, I.; Conto, F.D.; Chezzi, C.; Calderaro, A.; Ferri, C.; Caselli, E. Impact of Human Cytomegalovirus and Human Herpesvirus 6 Infection on the Expression of Factors Associated with Cell Fibrosis and Apoptosis: Clues for Implication in Systemic Sclerosis Development. *Int. J. Mol. Sci.* **2020**, *21*, 6397. [CrossRef] [PubMed]
104. Guo, G.; Ye, S.; Xie, S.; Ye, L.; Lin, C.; Yang, M.; Shi, X.; Wang, F.; Li, B.; Li, M.; et al. The Cytomegalovirus Protein US31 Induces Inflammation through Mono-Macrophages in Systemic Lupus Erythematosus by Promoting NF-KB2 Activation. *Cell Death Dis.* **2018**, *9*, 104. [CrossRef] [PubMed]
105. Milovanovic, J.; Popovic, B.; Milovanovic, M.; Kvestak, D.; Arsenijevic, A.; Stojanovic, B.; Tanaskovic, I.; Krmpotic, A.; Arsenijevic, N.; Jonjic, S.; et al. Murine Cytomegalovirus Infection Induces Susceptibility to EAE in Resistant BALB/c Mice. *Front. Immunol.* **2017**, *8*, 192. [CrossRef] [PubMed]
106. Wen, J.; Xiao, Y.; Wang, J.; Pan, W.; Zhou, Y.; Zhang, X.; Guan, W.; Chen, Y.; Zhou, K.; Wang, Y.; et al. Low Doses of CMV Induce Autoimmune-Mediated and Inflammatory Responses in Bile Duct Epithelia of Regulatory T Cell-Depleted Neonatal Mice. *Lab. Investig.* **2015**, *95*, 180–192. [CrossRef]

107. Zitti, B.; Bryceson, Y.T. Natural Killer Cells in Inflammation and Autoimmunity. *Cytokine Growth Factor Rev.* **2018**, *42*, 37–46. [CrossRef]
108. Martínez-Rodríguez, J.E.; Cobo-Calvo, A.; Villar, L.M.; Munteis, E.; Blanco, Y.; Rasal, R.; Vera, A.; Muntasell, A.; Alvarez-Lafuente, R.; Saiz, A.; et al. Adaptive Natural Killer Cell Response to Cytomegalovirus and Disability Progression in Multiple Sclerosis. *Mult. Scler.* **2016**, *22*, 741–752. [CrossRef]
109. McCarthy, N.E.; Eberl, M. Human Γδ T-Cell Control of Mucosal Immunity and Inflammation. *Front. Immunol.* **2018**, *9*. [CrossRef] [PubMed]
110. Tometten, I.; Felgentreff, K.; Hönig, M.; Hauck, F.; Albert, M.H.; Niehues, T.; Perez, R.; Ghosh, S.; Picard, C.; Stary, J.; et al. Increased Proportions of Γδ T Lymphocytes in Atypical SCID Associate with Disease Manifestations. *Clin. Immunol.* **2019**, *201*, 30–34. [CrossRef]
111. Lunardi, C.; Bason, C.; Corrocher, R.; Puccetti, A. Induction of Endothelial Cell Damage by HCMV Molecular Mimicry. *Trends Immunol.* **2005**, *26*, 19–24. [CrossRef]
112. Bason, C.; Corrocher, R.; Lunardi, C.; Puccetti, P.; Olivieri, O.; Girelli, D.; Navone, R.; Beri, R.; Millo, E.; Margonato, A.; et al. Interaction of Antibodies against Cytomegalovirus with Heat-Shock Protein 60 in Pathogenesis of Atherosclerosis. *Lancet* **2003**, *362*, 1971–1977. [CrossRef]
113. Dolcino, M.; Puccetti, A.; Barbieri, A.; Bason, C.; Tinazzi, E.; Ottria, A.; Patuzzo, G.; Martinelli, N.; Lunardi, C. Infections and Autoimmunity: Role of Human Cytomegalovirus in Autoimmune Endothelial Cell Damage. *Lupus* **2015**, *24*, 419–432. [CrossRef]
114. Broadley, I.; Pera, A.; Morrow, G.; Davies, K.A.; Kern, F. Expansions of Cytotoxic CD4+CD28- T Cells Drive Excess Cardiovascular Mortality in Rheumatoid Arthritis and Other Chronic Inflammatory Conditions and Are Triggered by CMV Infection. *Front. Immunol.* **2017**, *8*, 195. [CrossRef] [PubMed]
115. Pera, A.; Broadley, I.; Davies, K.A.; Kern, F. Cytomegalovirus as a Driver of Excess Cardiovascular Mortality in Rheumatoid Arthritis: A Red Herring or a Smoking Gun? *Circ. Res.* **2017**, *120*, 274–277. [CrossRef]
116. Di Battista, M.; Marcucci, E.; Elefante, E.; Tripoli, A.; Governato, G.; Zucchi, D.; Tani, C.; Alunno, A. One Year in Review 2018: Systemic Lupus Erythematosus. *Clin. Exp. Rheumatol.* **2018**, *36*, 763–777. [PubMed]
117. Luo, S.; Long, H.; Lu, Q. Recent Advances in Understanding Pathogenesis and Therapeutic Strategies of Systemic Lupus Erythematosus. *Int. Immunopharmacol.* **2020**, *89*, 107028. [CrossRef] [PubMed]
118. Pérez-Mercado, A.E.; Vilá-Pérez, S. Cytomegalovirus as a Trigger for Systemic Lupus Erythematosus. *J. Clin. Rheumatol.* **2010**, *16*, 335–337. [CrossRef]
119. Bendiksen, S.; Van Ghelue, M.; Rekvig, O.P.; Gutteberg, T.; Haga, H.J.; Moens, U. A Longitudinal Study of Human Cytomegalovirus Serology and Viruria Fails to Detect Active Viral Infection in 20 Systemic Lupus Erythematosus Patients. *Lupus* **2000**, *9*, 120–126. [CrossRef] [PubMed]
120. Rider, J.R.; Ollier, W.E.; Lock, R.J.; Brookes, S.T.; Pamphilon, D.H. Human Cytomegalovirus Infection and Systemic Lupus Erythematosus. *Clin. Exp. Rheumatol.* **1997**, *15*, 405–409.
121. Takizawa, Y.; Inokuma, S.; Tanaka, Y.; Saito, K.; Atsumi, T.; Hirakata, M.; Kameda, H.; Hirohata, S.; Kondo, H.; Kumagai, S.; et al. Clinical Characteristics of Cytomegalovirus Infection in Rheumatic Diseases: Multicentre Survey in a Large Patient Population. *Rheumatology* **2008**, *47*, 1373–1378. [CrossRef]
122. Newkirk, M.M.; van Venrooij, W.J.; Marshall, G.S. Autoimmune Response to U1 Small Nuclear Ribonucleoprotein (U1 SnRNP) Associated with Cytomegalovirus Infection. *Arthritis Res.* **2001**, *3*, 253–258. [CrossRef] [PubMed]
123. Su, B.Y.-J.; Su, C.-Y.; Yu, S.-F.; Chen, C.-J. Incidental Discovery of High Systemic Lupus Erythematosus Disease Activity Associated with Cytomegalovirus Viral Activity. *Med. Microbiol. Immunol.* **2007**, *196*, 165–170. [CrossRef] [PubMed]
124. McClain, M.T.; Poole, B.D.; Bruner, B.F.; Kaufman, K.M.; Harley, J.B.; James, J.A. An Altered Immune Response to Epstein-Barr Nuclear Antigen 1 in Pediatric Systemic Lupus Erythematosus. *Arthritis Rheum.* **2006**, *54*, 360–368. [CrossRef] [PubMed]
125. Parks, C.G.; Cooper, G.S.; Hudson, L.L.; Dooley, M.A.; Treadwell, E.L.; St.Clair, E.W.; Gilkeson, G.S.; Pandey, J.P. Association of Epstein-Barr Virus with Systemic Lupus Erythematosus: Effect Modification by Race, Age, and Cytotoxic T Lymphocyte–Associated Antigen 4 Genotype. *Arthritis Rheum.* **2005**, *52*, 1148–1159. [CrossRef]
126. James, J.A.; Neas, B.R.; Moser, K.L.; Hall, T.; Bruner, G.R.; Sestak, A.L.; Harley, J.B. Systemic Lupus Erythematosus in Adults Is Associated with Previous Epstein-Barr Virus Exposure. *Arthritis Rheum.* **2001**, *44*, 1122–1126. [CrossRef]
127. Harley, J.B.; James, J.A. Epstein-Barr Virus Infection Induces Lupus Autoimmunity. *Bull. Nyu Hosp. Jt Dis.* **2006**, *64*, 45–50. [PubMed]
128. Baboonian, C.; Venables, P.J.; Booth, J.; Williams, D.G.; Roffe, L.M.; Maini, R.N. Virus Infection Induces Redistribution and Membrane Localization of the Nuclear Antigen La (SS-B): A Possible Mechanism for Autoimmunity. *Clin. Exp. Immunol.* **1989**, *78*, 454–459.
129. Bouza, E.; Moya, J.G.; Muñoz, P. Infections in Systemic Lupus Erythematosus and Rheumatoid Arthritis. *Infect. Dis. Clin. North. Am.* **2001**, *15*, 335–361. [CrossRef]
130. Bertsias, G.; Ioannidis, J.P.A.; Boletis, J.; Bombardieri, S.; Cervera, R.; Dostal, C.; Font, J.; Gilboe, I.M.; Houssiau, F.; Huizinga, T.; et al. EULAR Recommendations for the Management of Systemic Lupus Erythematosus. Report of a Task Force of the EULAR Standing Committee for International Clinical Studies Including Therapeutics. *Ann. Rheum. Dis.* **2008**, *67*, 195–205. [CrossRef] [PubMed]
131. Fei, Y.; Shi, X.; Gan, F.; Li, X.; Zhang, W.; Li, M.; Hou, Y.; Zhang, X.; Zhao, Y.; Zeng, X.; et al. Death Causes and Pathogens Analysis of Systemic Lupus Erythematosus during the Past 26 Years. *Clin. Rheumatol.* **2014**, *33*, 57–63. [CrossRef]

132. Choo, H.M.C.; Cher, W.Q.; Kwan, Y.H.; Fong, W.W.S. Risk Factors for Cytomegalovirus Disease in Systemic Lupus Erythematosus (SLE): A Systematic Review. *Adv. Rheumatol.* **2019**, *59*, 12. [CrossRef]
133. Xue, Y.; Jiang, L.; Wan, W.-G.; Chen, Y.-M.; Zhang, J.; Zhang, Z.-C. Cytomegalovirus Pneumonia in Patients with Rheumatic Diseases After Immunosuppressive Therapy: A Single Center Study in China. *Chin. Med. J.* **2016**, *129*, 267–273. [CrossRef]
134. Qin, L.; Qiu, Z.; Hsieh, E.; Geng, T.; Zhao, J.; Zeng, X.; Wan, L.; Xie, J.; Ramendra, R.; Routy, J.P.; et al. Association between Lymphocyte Subsets and Cytomegalovirus Infection Status among Patients with Systemic Lupus Erythematosus: A Pilot Study. *Medicine* **2019**, *98*, e16997. [CrossRef] [PubMed]
135. Elhai, M.; Meune, C.; Avouac, J.; Kahan, A.; Allanore, Y. Trends in Mortality in Patients with Systemic Sclerosis over 40 Years: A Systematic Review and Meta-Analysis of Cohort Studies. *Rheumatology* **2012**, *51*, 1017–1026. [CrossRef] [PubMed]
136. Marie, I.; Gehanno, J.-F. Environmental Risk Factors of Systemic Sclerosis. *Semin. Immunopathol.* **2015**, *37*, 463–473. [CrossRef]
137. Gyftaki-Venieri, D.A.; Abraham, D.J.; Ponticos, M. Insights into Myofibroblasts and Their Activation in Scleroderma: Opportunities for Therapy? *Curr. Opin. Rheumatol.* **2018**, *30*, 581–587. [CrossRef]
138. Harvey, G.R.; McHugh, N.J. Serologic Abnormalities in Systemic Sclerosis. *Curr. Opin. Rheumatol.* **1999**, *11*, 495–502. [CrossRef] [PubMed]
139. Radić, M.; Martinović Kaliterna, D.; Radić, J. Infectious Disease as Aetiological Factor in the Pathogenesis of Systemic Sclerosis. *Neth. J. Med.* **2010**, *68*, 348–353.
140. Randone, S.B.; Guiducci, S.; Cerinic, M.M. Systemic Sclerosis and Infections. *Autoimmun. Rev.* **2008**, *8*, 36–40. [CrossRef]
141. Bilgin, H.; Kocabaş, H.; Keşli, R. The Prevalence of Infectious Agents in Patients with Systemic Sclerosis. *Turk. J. Med. Sci.* **2015**, *45*, 1192–1197. [CrossRef] [PubMed]
142. Trombetta, A.C.; Tomatis, V.; Alessandri, E.; Paolino, S.; Pizzorni, C.; Ghio, M.; Ruaro, B.; Patane, M.; Gotelli, E.; Goegan, F.; et al. AB0723 Seroprevalence of Epstein-Barr Virus and Cytomegalovirus in Systemic Sclerosis Patients: Preliminary Results. *Ann. Rheum. Dis.* **2018**, *77*, 1500–1501. [CrossRef]
143. Esen, B.A.; Yılmaz, G.; Uzun, S.; Ozdamar, M.; Aksözek, A.; Kamalı, S.; Türkoğlu, S.; Gül, A.; Ocal, L.; Aral, O.; et al. Serologic Response to Epstein-Barr Virus Antigens in Patients with Systemic Lupus Erythematosus: A Controlled Study. *Rheumatol. Int.* **2012**, *32*, 79–83. [CrossRef] [PubMed]
144. Neidhart, M.; Kuchen, S.; Distler, O.; Brühlmann, P.; Michel, B.A.; Gay, R.E.; Gay, S. Increased Serum Levels of Antibodies against Human Cytomegalovirus and Prevalence of Autoantibodies in Systemic Sclerosis. *Arthritis Rheum.* **1999**, *42*, 389–392. [CrossRef]
145. Pandey, J.P. Immunoglobulin GM Genes and IgG Antibodies to Cytomegalovirus in Patients with Systemic Sclerosis. *Clin. Exp. Rheumatol.* **2004**, *22*, S35–S37.
146. Arnson, Y.; Amital, H.; Guiducci, S.; Matucci-Cerinic, M.; Valentini, G.; Barzilai, O.; Maya, R.; Shoenfeld, Y. The Role of Infections in the Immunopathogensis of Systemic Sclerosis–Evidence from Serological Studies. *Ann. N. Y. Acad. Sci.* **2009**, *1173*, 627–632. [CrossRef] [PubMed]
147. Vaughan, J.H.; Shaw, P.X.; Nguyen, M.D.; Medsger, T.A.; Wright, T.M.; Metcalf, J.S.; Leroy, E.C. Evidence of Activation of 2 Herpesviruses, Epstein-Barr Virus and Cytomegalovirus, in Systemic Sclerosis and Normal Skins. *J. Rheumatol.* **2000**, *27*, 821–823. [PubMed]
148. Jarvis, M.A.; Nelson, J.A. Human Cytomegalovirus Persistence and Latency in Endothelial Cells and Macrophages. *Curr. Opin. Microbiol.* **2002**, *5*, 403–407. [CrossRef]
149. Efthymiou, G.; Dardiotis, E.; Liaskos, C.; Marou, E.; Scheper, T.; Meyer, W.; Daponte, A.; Daoussis, D.; Hadjigeorgiou, G.; Bogdanos, D.P.; et al. A Comprehensive Analysis of Antigen-Specific Antibody Responses against Human Cytomegalovirus in Patients with Systemic Sclerosis. *Clin. Immunol.* **2019**, *207*, 87–96. [CrossRef]
150. Balandraud, N.; Roudier, J. Epstein-Barr Virus and Rheumatoid Arthritis. *Jt. Bone Spine* **2018**, *85*, 165–170. [CrossRef] [PubMed]
151. Musiani, M.; Zerbini, M.; Ferri, S.; Plazzi, M.; Gentilomi, G.; La Placa, M. Comparison of the Immune Response to Epstein-Barr Virus and Cytomegalovirus in Sera and Synovial Fluids of Patients with Rheumatoid Arthritis. *Ann. Rheum. Dis.* **1987**, *46*, 837–842. [CrossRef]
152. Bassyouni, R.H.; Dwedar, R.A.; Ezzat, E.M.; Marzaban, R.N.; Nassr, M.H.; Rashid, L. Elevated Cytomegalovirus and Epstein-Barr Virus Burden in Rheumatoid Arthritis: A True Pathogenic Role or Just a Coincidence. *Egypt. Rheumatol.* **2019**, *41*, 255–259. [CrossRef]
153. Pierer, M.; Rothe, K.; Quandt, D.; Schulz, A.; Rossol, M.; Scholz, R.; Baerwald, C.; Wagner, U. Association of Anticytomegalovirus Seropositivity with More Severe Joint Destruction and More Frequent Joint Surgery in Rheumatoid Arthritis. *Arthritis Rheum.* **2012**, *64*, 1740–1749. [CrossRef] [PubMed]
154. Alvarez-Lafuente, R.; Fernández-Gutiérrez, B.; de Miguel, S.; Jover, J.A.; Rollin, R.; Loza, E.; Clemente, D.; Lamas, J.R. Potential Relationship between Herpes Viruses and Rheumatoid Arthritis: Analysis with Quantitative Real Time Polymerase Chain Reaction. *Ann. Rheum. Dis.* **2005**, *64*, 1357–1359. [CrossRef] [PubMed]
155. Mourgues, C.; Henquell, C.; Tatar, Z.; Pereira, B.; Nourisson, C.; Tournadre, A.; Soubrier, M.; Couderc, M. Monitoring of Epstein-Barr Virus (EBV)/Cytomegalovirus (CMV)/Varicella-Zoster Virus (VZV) Load in Patients Receiving Tocilizumab for Rheumatoid Arthritis. *Jt. Bone Spine* **2016**, *83*, 412–415. [CrossRef] [PubMed]
156. Mehraein, Y.; Lennerz, C.; Ehlhardt, S.; Remberger, K.; Ojak, A.; Zang, K.D. Latent Epstein-Barr Virus (EBV) Infection and Cytomegalovirus (CMV) Infection in Synovial Tissue of Autoimmune Chronic Arthritis Determined by RNA- and DNA-in Situ Hybridization. *Mod. Pathol.* **2004**, *17*, 781–789. [CrossRef] [PubMed]

157. Garrod, A.B. On Gout and Rheumatism. The Differential Diagnosis, and the Nature of the so-Called Rheumatic Gout. *Med. Chir. Trans.* **1854**, *37*, 181–220. [CrossRef] [PubMed]
158. Silman, A.J.; Pearson, J.E. Epidemiology and Genetics of Rheumatoid Arthritis. *Arthritis Res.* **2002**, *4* (Suppl. 3), S265–S272. [CrossRef]
159. Wolfe, A.M.; Kellgren, J.H.; Masi, A.T. The Epidemiology of Rheumatoid Arthritis: A Review. II. Incidence and Diagnostic Criteria. *Bull. Rheum Dis.* **1968**, *19*, 524–529. [PubMed]
160. Firestein, G.S. Evolving Concepts of Rheumatoid Arthritis. *Nature* **2003**, *423*, 356–361. [CrossRef]
161. Cojocaru, M.; Cojocaru, I.M.; Silosi, I.; Vrabie, C.D.; Tanasescu, R. Extra-Articular Manifestations in Rheumatoid Arthritis. *Maedica* **2010**, *5*, 286–291.
162. Taylor, P.; Gartemann, J.; Hsieh, J.; Creeden, J. A Systematic Review of Serum Biomarkers Anti-Cyclic Citrullinated Peptide and Rheumatoid Factor as Tests for Rheumatoid Arthritis. *Autoimmune Dis.* **2011**, *2011*, 815038. [CrossRef] [PubMed]
163. Nishimura, K.; Sugiyama, D.; Kogata, Y.; Tsuji, G.; Nakazawa, T.; Kawano, S.; Saigo, K.; Morinobu, A.; Koshiba, M.; Kuntz, K.M.; et al. Meta-Analysis: Diagnostic Accuracy of Anti-Cyclic Citrullinated Peptide Antibody and Rheumatoid Factor for Rheumatoid Arthritis. *Ann. Intern. Med.* **2007**, *146*, 797–808. [CrossRef] [PubMed]
164. Bizzaro, N.; Bartoloni, E.; Morozzi, G.; Manganelli, S.; Riccieri, V.; Sabatini, P.; Filippini, M.; Tampoia, M.; Afeltra, A.; Sebastiani, G.; et al. Anti-Cyclic Citrullinated Peptide Antibody Titer Predicts Time to Rheumatoid Arthritis Onset in Patients with Undifferentiated Arthritis: Results from a 2-Year Prospective Study. *Arthritis Res.* **2013**, *15*, R16. [CrossRef]
165. Vossenaar, E.R.; van Venrooij, W.J. Citrullinated Proteins: Sparks That May Ignite the Fire in Rheumatoid Arthritis. *Arthritis Res.* **2004**, *6*, 107–111. [CrossRef]
166. Acharya, N.K.; Nagele, E.P.; Han, M.; Coretti, N.J.; DeMarshall, C.; Kosciuk, M.C.; Boulos, P.A.; Nagele, R.G. Neuronal PAD4 Expression and Protein Citrullination: Possible Role in Production of Autoantibodies Associated with Neurodegenerative Disease. *J. Autoimmun.* **2012**, *38*, 369–380. [CrossRef]
167. Knight, J.S.; Subramanian, V.; O'Dell, A.A.; Yalavarthi, S.; Zhao, W.; Smith, C.K.; Hodgin, J.B.; Thompson, P.R.; Kaplan, M.J. Peptidylarginine Deiminase Inhibition Disrupts NET Formation and Protects against Kidney, Skin and Vascular Disease in Lupus-Prone MRL/Lpr Mice. *Ann. Rheum. Dis.* **2015**, *74*, 2199–2206. [CrossRef]
168. Yang, L.; Tan, D.; Piao, H. Myelin Basic Protein Citrullination in Multiple Sclerosis: A Potential Therapeutic Target for the Pathology. *Neurochem. Res.* **2016**, *41*, 1845–1856. [CrossRef] [PubMed]
169. Yuzhalin, A.E. Citrullination in Cancer. *Cancer Res.* **2019**, *79*, 1274–1284. [CrossRef] [PubMed]
170. Suzuki, A.; Yamada, R.; Chang, X.; Tokuhiro, S.; Sawada, T.; Suzuki, M.; Nagasaki, M.; Nakayama-Hamada, M.; Kawaida, R.; Ono, M.; et al. Functional Haplotypes of PADI4, Encoding Citrullinating Enzyme Peptidylarginine Deiminase 4, Are Associated with Rheumatoid Arthritis. *Nat. Genet.* **2003**, *34*, 395–402. [CrossRef]
171. Kuhn, K.A.; Kulik, L.; Tomooka, B.; Braschler, K.J.; Arend, W.P.; Robinson, W.H.; Holers, V.M. Antibodies against Citrullinated Proteins Enhance Tissue Injury in Experimental Autoimmune Arthritis. *J. Clin. Investig.* **2006**, *116*, 961–973. [CrossRef] [PubMed]
172. Foulquier, C.; Sebbag, M.; Clavel, C.; Chapuy-Regaud, S.; Al Badine, R.; Méchin, M.-C.; Vincent, C.; Nachat, R.; Yamada, M.; Takahara, H.; et al. Peptidyl Arginine Deiminase Type 2 (PAD-2) and PAD-4 but Not PAD-1, PAD-3, and PAD-6 Are Expressed in Rheumatoid Arthritis Synovium in Close Association with Tissue Inflammation. *Arthritis Rheum.* **2007**, *56*, 3541–3553. [CrossRef]
173. Willis, V.C.; Gizinski, A.M.; Banda, N.K.; Causey, C.P.; Knuckley, B.; Cordova, K.N.; Luo, Y.; Levitt, B.; Glogowska, M.; Chandra, P.; et al. N-α-Benzoyl-N5-(2-Chloro-1-Iminoethyl)-L-Ornithine Amide, a Protein Arginine Deiminase Inhibitor, Reduces the Severity of Murine Collagen-Induced Arthritis. *J. Immunol.* **2011**, *186*, 4396–4404. [CrossRef]
174. Klareskog, L.; Stolt, P.; Lundberg, K.; Källberg, H.; Bengtsson, C.; Grunewald, J.; Rönnelid, J.; Harris, H.E.; Ulfgren, A.-K.; Rantapää-Dahlqvist, S.; et al. A New Model for an Etiology of Rheumatoid Arthritis: Smoking May Trigger HLA-DR (Shared Epitope)-Restricted Immune Reactions to Autoantigens Modified by Citrullination. *Arthritis Rheum.* **2006**, *54*, 38–46. [CrossRef] [PubMed]
175. Deo, S.S.; Shetty, R.R.; Mistry, K.J.; Chogle, A.R. Detection of Viral Citrullinated Peptide Antibodies Directed Against EBV or VCP: In Early Rheumatoid Arthritis Patients of Indian Origin. *J. Lab. Physicians* **2010**, *2*, 93–99. [CrossRef]
176. Kraal, L.J.N.; Nijland, M.L.; Germar, K.L.; Baeten, D.L.P.; ten Berge, I.J.M.; Fehres, C.M. Anti-Citrullinated Protein Antibody Response after Primary EBV Infection in Kidney Transplant Patients. *PLoS ONE* **2018**, *13*, e0197219. [CrossRef]
177. Trier, N.H.; Holm, B.E.; Heiden, J.; Slot, O.; Locht, H.; Lindegaard, H.; Svendsen, A.; Nielsen, C.T.; Jacobsen, S.; Theander, E.; et al. Antibodies to a Strain-Specific Citrullinated Epstein-Barr Virus Peptide Diagnoses Rheumatoid Arthritis. *Sci. Rep.* **2018**, *8*, 3684. [CrossRef] [PubMed]
178. Casanova, V.; Sousa, F.H.; Shakamuri, P.; Svoboda, P.; Buch, C.; D'Acremont, M.; Christophorou, M.A.; Pohl, J.; Stevens, C.; Barlow, P.G. Citrullination Alters the Antiviral and Immunomodulatory Activities of the Human Cathelicidin LL-37 During Rhinovirus Infection. *Front. Immunol.* **2020**, *11*. [CrossRef] [PubMed]
179. Davis, J.M.; Knutson, K.L.; Skinner, J.A.; Strausbauch, M.A.; Crowson, C.S.; Therneau, T.M.; Wettstein, P.J.; Matteson, E.L.; Gabriel, S.E. A Profile of Immune Response to Herpesvirus Is Associated with Radiographic Joint Damage in Rheumatoid Arthritis. *Arthritis Res.* **2012**, *14*, R24. [CrossRef]
180. Martens, P.B.; Goronzy, J.J.; Schaid, D.; Weyand, C.M. Expansion of Unusual CD4+ T Cells in Severe Rheumatoid Arthritis. *Arthritis Rheum.* **1997**, *40*, 1106–1114. [CrossRef] [PubMed]

181. Pawlik, A.; Ostanek, L.; Brzosko, I.; Brzosko, M.; Masiuk, M.; Machalinski, B.; Gawronska-Szklarz, B. The Expansion of CD4+CD28− T Cells in Patients with Rheumatoid Arthritis. *Arthritis Res.* **2003**, *5*, R210–R213. [CrossRef]
182. Namekawa, T.; Wagner, U.G.; Goronzy, J.J.; Weyand, C.M. Functional Subsets of CD4 T Cells in Rheumatoid Synovitis. *Arthritis Rheum.* **1998**, *41*, 2108–2116. [CrossRef]
183. Hooper, M.; Kallas, E.G.; Coffin, D.; Campbell, D.; Evans, T.G.; Looney, R.J. Cytomegalovirus Seropositivity Is Associated with the Expansion of CD4+CD28− and CD8+CD28− T Cells in Rheumatoid Arthritis. *J. Rheumatol.* **1999**, *26*, 1452–1457.
184. Lamprecht, P.; Gross, W.L. Wegener's Granulomatosis. *Herz* **2004**, *29*, 47–56. [CrossRef]
185. Hamerman, D.; Gresser, I.; Smith, C. Isolation of Cytomegalovirus from Synovial Cells of a Patient with Rheumatoid Arthritis. *J. Rheumatol.* **1982**, *9*, 658–664. [PubMed]
186. Einsele, H.; Steidle, M.; Müller, C.A.; Fritz, P.; Zacher, J.; Schmidt, H.; Saal, J.G. Demonstration of Cytomegalovirus (CMV) DNA and Anti-CMV Response in the Synovial Membrane and Serum of Patients with Rheumatoid Arthritis. *J. Rheumatol.* **1992**, *19*, 677–681.
187. Murayama, T.; Jisaki, F.; Ayata, M.; Sakamuro, D.; Hironaka, T.; Hirai, K.; Tsuchiya, N.; Ito, K.; Furukawa, T. Cytomegalovirus Genomes Demonstrated by Polymerase Chain Reaction in Synovial Fluid from Rheumatoid Arthritis Patients. *Clin. Exp. Rheumatol.* **1992**, *10*, 161–164.
188. Xu, X.; Estekizadeh, A.; Davoudi, B.; Varani, S.; Malmström, V.; Rahbar, A.; Söderberg-Nauclér, C. Detection of Human Cytomegalovirus in Synovial Neutrophils Obtained from Patients with Rheumatoid Arthritis. *Scand. J. Rheumatol.* **2020**, 1–6. [CrossRef]
189. Melnick, J.L.; Hu, C.; Burek, J.; Adam, E.; DeBakey, M.E. Cytomegalovirus DNA in Arterial Walls of Patients with Atherosclerosis. *J. Med. Virol.* **1994**, *42*, 170–174. [CrossRef]
190. Xenaki, E.; Hassoulas, J.; Apostolakis, S.; Sourvinos, G.; Spandidos, D.A. Detection of Cytomegalovirus in Atherosclerotic Plaques and Nonatherosclerotic Arteries. *Angiology* **2009**, *60*, 504–508. [CrossRef] [PubMed]
191. Wang, H.; Peng, G.; Bai, J.; He, B.; Huang, K.; Hu, X.; Liu, D. Cytomegalovirus Infection and Relative Risk of Cardiovascular Disease (Ischemic Heart Disease, Stroke, and Cardiovascular Death): A Meta-Analysis of Prospective Studies Up to 2016. *J. Am. Heart Assoc.* **2017**, *6*. [CrossRef] [PubMed]
192. Shenk, T.; Alwine, J.C. Human Cytomegalovirus: Coordinating Cellular Stress, Signaling, and Metabolic Pathways. *Annu. Rev. Virol.* **2014**, *1*, 355–374. [CrossRef]
193. Northfield, J.; Lucas, M.; Jones, H.; Young, N.T.; Klenerman, P. Does Memory Improve with Age? CD85j (ILT-2/LIR-1) Expression on CD8 T Cells Correlates with "memory Inflation" in Human Cytomegalovirus Infection. *Immunol. Cell Biol.* **2005**, *83*, 182–188. [CrossRef] [PubMed]
194. Wagner, C.S.; Walther-Jallow, L.; Buentke, E.; Ljunggren, H.-G.; Achour, A.; Chambers, B.J. Human Cytomegalovirus-Derived Protein UL18 Alters the Phenotype and Function of Monocyte-Derived Dendritic Cells. *J. Leukoc. Biol.* **2008**, *83*, 56–63. [CrossRef]
195. Saverino, D.; Fabbi, M.; Ghiotto, F.; Merlo, A.; Bruno, S.; Zarcone, D.; Tenca, C.; Tiso, M.; Santoro, G.; Anastasi, G.; et al. The CD85/LIR-1/ILT2 Inhibitory Receptor Is Expressed by All Human T Lymphocytes and down-Regulates Their Functions. *J. Immunol.* **2000**, *165*, 3742–3755. [CrossRef] [PubMed]
196. Compston, A.; Coles, A. Multiple Sclerosis. *Lancet* **2008**, *372*, 1502–1517. [CrossRef]
197. Olsson, T.; Barcellos, L.F.; Alfredsson, L. Interactions between Genetic, Lifestyle and Environmental Risk Factors for Multiple Sclerosis. *Nat. Rev. Neurol.* **2017**, *13*, 25–36. [CrossRef]
198. Lucas, R.M.; Hughes, A.M.; Lay, M.-L.J.; Ponsonby, A.-L.; Dwyer, D.E.; Taylor, B.V.; Pender, M.P. Epstein-Barr Virus and Multiple Sclerosis. *J. Neurol. Neurosurg. Psychiatry* **2011**, *82*, 1142–1148. [CrossRef]
199. Ascherio, A. Environmental Factors in Multiple Sclerosis. *Expert Rev. Neurother.* **2013**, *13*, 3–9. [CrossRef]
200. Alari-Pahissa, E.; Moreira, A.; Zabalza, A.; Alvarez-Lafuente, R.; Munteis, E.; Vera, A.; Arroyo, R.; Alvarez-Cermeño, J.C.; Villar, L.M.; López-Botet, M.; et al. Low Cytomegalovirus Seroprevalence in Early Multiple Sclerosis: A Case for the 'Hygiene Hypothesis'? *Eur. J. Neurol.* **2018**, *25*, 925–933. [CrossRef]
201. White, D.W.; Suzanne Beard, R.; Barton, E.S. Immune Modulation during Latent Herpesvirus Infection. *Immunol. Rev.* **2012**, *245*, 189–208. [CrossRef]
202. Sanadgol, N.; Ramroodi, N.; Ahmadi, G.A.; Komijani, M.; Moghtaderi, A.; Bouzari, M.; Rezaei, M.; Kardi, M.T.; Dabiri, S.; Moradi, M.; et al. Prevalence of Cytomegalovirus Infection and Its Role in Total Immunoglobulin Pattern in Iranian Patients with Different Subtypes of Multiple Sclerosis. *New Microbiol.* **2011**, *34*, 263–274. [PubMed]
203. Najafi, S.; Ghane, M.; Poortahmasebi, V.; Jazayeri, S.M.; Yousefzadeh-Chabok, S. Prevalence of Cytomegalovirus in Patients With Multiple Sclerosis: A Case-Control Study in Northern Iran. *Jundishapur J. Microbiol.* **2016**, *9*, e36582. [CrossRef]
204. Vanheusden, M.; Stinissen, P.; 't Hart, B.A.; Hellings, N. Cytomegalovirus: A Culprit or Protector in Multiple Sclerosis? *Trends Mol. Med.* ***2015***, 21, *16–23. [1.002*CrossRef] [PubMed]
205. Clerico, M.; De Mercanti, S.; Artusi, C.A.; Durelli, L.; Naismith, R.T. Active CMV Infection in Two Patients with Multiple Sclerosis Treated with Alemtuzumab. *Mult. Scler.* **2017**, *23*, 874–876. [CrossRef] [PubMed]
206. Costa-Garcia, M.; Vera, A.; Moraru, M.; Vilches, C.; López-Botet, M.; Muntasell, A. Antibody-Mediated Response of NKG2Cbright NK Cells against Human Cytomegalovirus. *J. Immunol.* **2015**, *194*, 2715–2724. [CrossRef] [PubMed]

207. López-Montañés, M.; Alari-Pahissa, E.; Sintes, J.; Martínez-Rodríguez, J.E.; Muntasell, A.; López-Botet, M. Antibody-Dependent NK Cell Activation Differentially Targets EBV-Infected Cells in Lytic Cycle and Bystander B Lymphocytes Bound to Viral Antigen-Containing Particles. *J. Immunol.* **2017**, *199*, 656–665. [CrossRef] [PubMed]
208. Lünemann, J.D.; Tintoré, M.; Messmer, B.; Strowig, T.; Rovira, A.; Perkal, H.; Caballero, E.; Münz, C.; Montalban, X.; Comabella, M. Elevated Epstein-Barr Virus-Encoded Nuclear Antigen-1 Immune Responses Predict Conversion to Multiple Sclerosis. *Ann. Neurol.* **2010**, *67*, 159–169. [CrossRef]
209. Maple, P.A.C.; Tanasescu, R.; Gran, B.; Constantinescu, C.S. A Different Response to Cytomegalovirus (CMV) and Epstein-Barr Virus (EBV) Infection in UK People with Multiple Sclerosis (PwMS) Compared to Controls. *J. Infect.* **2020**, *80*, 320–325. [CrossRef]
210. Baroco, A.L.; Oldfield, E.C. Gastrointestinal Cytomegalovirus Disease in the Immunocompromised Patient. *Curr. Gastroenterol. Rep.* **2008**, *10*, 409–416. [CrossRef]
211. Esclatine, A.; Lemullois, M.; Servin, A.L.; Quero, A.M.; Geniteau-Legendre, M. Human Cytomegalovirus Infects Caco-2 Intestinal Epithelial Cells Basolaterally Regardless of the Differentiation State. *J. Virol.* **2000**, *74*, 513–517. [CrossRef]
212. Jarvis, M.A.; Wang, C.E.; Meyers, H.L.; Smith, P.P.; Corless, C.L.; Henderson, G.J.; Vieira, J.; Britt, W.J.; Nelson, J.A. Human Cytomegalovirus Infection of Caco-2 Cells Occurs at the Basolateral Membrane and Is Differentiation State Dependent. *J. Virol.* **1999**, *73*, 4552–4560. [CrossRef]
213. Nagai, S.; Mangus, R.S.; Anderson, E.; Ekser, B.; Kubal, C.A.; Fridell, J.A.; Tector, A.J. Cytomegalovirus Infection After Intestinal/Multivisceral Transplantation: A Single-Center Experience With 210 Cases. *Transplantation* **2016**, *100*, 451–460. [CrossRef]
214. Chiereghin, A.; Gabrielli, L.; Zanfi, C.; Petrisli, E.; Lauro, A.; Piccirilli, G.; Baccolini, F.; Dazzi, A.; Cescon, M.; Morelli, M.C.; et al. Monitoring Cytomegalovirus T-Cell Immunity in Small Bowel/Multivisceral Transplant Recipients. *Transplant. Proc.* **2010**, *42*, 69–73. [CrossRef]
215. Varani, S.; Landini, M.P. Cytomegalovirus-Induced Immunopathology and Its Clinical Consequences. *Herpesviridae* **2011**, *2*, 6. [CrossRef]
216. Ramos, G.P.; Papadakis, K.A. Mechanisms of Disease: Inflammatory Bowel Diseases. *Mayo Clin. Proc.* **2019**, *94*, 155–165. [CrossRef]
217. Jentzer, A.; Veyrard, P.; Roblin, X.; Saint-Sardos, P.; Rochereau, N.; Paul, S.; Bourlet, T.; Pozzetto, B.; Pillet, S. Cytomegalovirus and Inflammatory Bowel Diseases (IBD) with a Special Focus on the Link with Ulcerative Colitis (UC). *Microorganisms* **2020**, *8*, 1078. [CrossRef] [PubMed]
218. Strober, W.; Fuss, I.J. Proinflammatory Cytokines in the Pathogenesis of Inflammatory Bowel Diseases. *Gastroenterology* **2011**, *140*, 1756–1767. [CrossRef]
219. Powell, R.D.; Warner, N.E.; Levine, R.S.; Kirsner, J.B. Cytomegalic Inclusion Disease and Ulcerative Colitis: Report of a Case in a Young Adult. *Am. J. Med.* **1961**, *30*, 334–340. [CrossRef]
220. Hendler, S.A.; Barber, G.E.; Okafor, P.N.; Chang, M.S.; Limsui, D.; Limketkai, B.N. Cytomegalovirus Infection Is Associated with Worse Outcomes in Inflammatory Bowel Disease Hospitalizations Nationwide. *Int. J. Colorectal. Dis.* **2020**, *35*, 897–903. [CrossRef]
221. Taylor-Wiedeman, J.; Sissons, J.G.; Borysiewicz, L.K.; Sinclair, J.H. Monocytes Are a Major Site of Persistence of Human Cytomegalovirus in Peripheral Blood Mononuclear Cells. *J. Gen. Virol.* **1991**, *72*, 2059–2064. [CrossRef] [PubMed]
222. Söderberg-Nauclér, C.; Fish, K.N.; Nelson, J.A. Reactivation of Latent Human Cytomegalovirus by Allogeneic Stimulation of Blood Cells from Healthy Donors. *Cell* **1997**, *91*, 119–126. [CrossRef]
223. Reeves, M.B.; Lehner, P.J.; Sissons, J.G.P.; Sinclair, J.H. An in Vitro Model for the Regulation of Human Cytomegalovirus Latency and Reactivation in Dendritic Cells by Chromatin Remodelling. *J. Gen. Virol.* **2005**, *86*, 2949–2954. [CrossRef] [PubMed]
224. Hommes, D.W.; Sterringa, G.; van Deventer, S.J.H.; Tytgat, G.N.J.; Weel, J. The Pathogenicity of Cytomegalovirus in Inflammatory Bowel Disease: A Systematic Review and Evidence-Based Recommendations for Future Research. *Inflamm. Bowel. Dis.* **2004**, *10*, 245–250. [CrossRef]
225. Julka, K.; Surawicz, C.M. Cytomegalovirus in Inflammatory Bowel Disease: Time for Another Look? *Gastroenterology* **2009**, *137*, 1163–1166. [CrossRef] [PubMed]
226. Kuwabara, A.; Okamoto, H.; Suda, T.; Ajioka, Y.; Hatakeyama, K. Clinicopathologic Characteristics of Clinically Relevant Cytomegalovirus Infection in Inflammatory Bowel Disease. *J. Gastroenterol.* **2007**, *42*, 823–829. [CrossRef]
227. Lawlor, G.; Moss, A.C. Cytomegalovirus in Inflammatory Bowel Disease: Pathogen or Innocent Bystander? *Inflamm. Bowel. Dis.* **2010**, *16*, 1620–1627. [CrossRef]
228. Pillet, S.; Pozzetto, B.; Roblin, X. Cytomegalovirus and Ulcerative Colitis: Place of Antiviral Therapy. *World J. Gastroenterol.* **2016**, *22*, 2030–2045. [CrossRef] [PubMed]
229. Mourad, F.H.; Hashash, J.G.; Kariyawasam, V.C.; Leong, R.W. Ulcerative Colitis and Cytomegalovirus Infection: From A to Z. *J. Crohns Colitis* **2020**, *14*, 1162–1171. [CrossRef]
230. Matsuoka, K.; Iwao, Y.; Mori, T.; Sakuraba, A.; Yajima, T.; Hisamatsu, T.; Okamoto, S.; Morohoshi, Y.; Izumiya, M.; Ichikawa, H.; et al. Cytomegalovirus Is Frequently Reactivated and Disappears without Antiviral Agents in Ulcerative Colitis Patients. *Am. J. Gastroenterol.* **2007**, *102*, 331–337. [CrossRef]
231. Domènech, E.; Vega, R.; Ojanguren, I.; Hernández, A.; Garcia-Planella, E.; Bernal, I.; Rosinach, M.; Boix, J.; Cabré, E.; Gassull, M.A. Cytomegalovirus Infection in Ulcerative Colitis: A Prospective, Comparative Study on Prevalence and Diagnostic Strategy. *Inflamm. Bowel Dis.* **2008**, *14*, 1373–1379. [CrossRef]

232. Lv, Y.-L.; Han, F.-F.; Jia, Y.-J.; Wan, Z.-R.; Gong, L.-L.; Liu, H.; Liu, L.-H. Is Cytomegalovirus Infection Related to Inflammatory Bowel Disease, Especially Steroid-Resistant Inflammatory Bowel Disease? A Meta-Analysis. *Infect. Drug Resist.* **2017**, *10*, 511–519. [CrossRef] [PubMed]
233. Hissong, E.; Chen, Z.; Yantiss, R.K. Cytomegalovirus Reactivation in Inflammatory Bowel Disease: An Uncommon Occurrence Related to Corticosteroid Dependence. *Mod. Pathol.* **2019**, *32*, 1210–1216. [CrossRef] [PubMed]
234. Criscuoli, V.; Rizzuto, M.-R.; Cottone, M. Cytomegalovirus and Inflammatory Bowel Disease: Is There a Link? *World J. Gastroenterol.* **2006**, *12*, 4813–4818. [CrossRef] [PubMed]
235. Rahbar, A.; Boström, L.; Lagerstedt, U.; Magnusson, I.; Söderberg-Naucler, C.; Sundqvist, V.-A. Evidence of Active Cytomegalovirus Infection and Increased Production of IL-6 in Tissue Specimens Obtained from Patients with Inflammatory Bowel Diseases. *Inflamm. Bowel Dis.* **2003**, *9*, 154–161. [CrossRef] [PubMed]
236. Kim, J.J.; Simpson, N.; Klipfel, N.; Debose, R.; Barr, N.; Laine, L. Cytomegalovirus Infection in Patients with Active Inflammatory Bowel Disease. *Dig. Dis. Sci.* **2010**, *55*, 1059–1065. [CrossRef]
237. Dimitroulia, E.; Spanakis, N.; Konstantinidou, A.E.; Legakis, N.J.; Tsakris, A. Frequent Detection of Cytomegalovirus in the Intestine of Patients with Inflammatory Bowel Disease. *Inflamm. Bowel Dis.* **2006**, *12*, 879–884. [CrossRef]
238. Roblin, X.; Pillet, S.; Berthelot, P.; Del Tedesco, E.; Phelip, J.-M.; Chambonnière, M.-L.; Peyrin-Biroulet, L.; Pozzetto, B. Prevalence of Cytomegalovirus Infection in Steroid-Refractory Crohn's Disease. *Inflamm. Bowel Dis.* **2012**, *18*, E1396–E1397. [CrossRef]
239. Römkens, T.E.H.; Bulte, G.J.; Nissen, L.H.C.; Drenth, J.P.H. Cytomegalovirus in Inflammatory Bowel Disease: A Systematic Review. *World J. Gastroenterol.* **2016**, *22*, 1321–1330. [CrossRef]
240. Knösel, T.; Schewe, C.; Petersen, N.; Dietel, M.; Petersen, I. Prevalence of Infectious Pathogens in Crohn's Disease. *Pathol. Res. Pract.* **2009**, *205*, 223–230. [CrossRef]
241. Takahashi, Y.; Tange, T. Prevalence of Cytomegalovirus Infection in Inflammatory Bowel Disease Patients. *Dis. Colon Rectum.* **2004**, *47*, 722–726. [CrossRef] [PubMed]
242. Papadakis, K.A.; Tung, J.K.; Binder, S.W.; Kam, L.Y.; Abreu, M.T.; Targan, S.R.; Vasiliauskas, E.A. Outcome of Cytomegalovirus Infections in Patients with Inflammatory Bowel Disease. *Am. J. Gastroenterol.* **2001**, *96*, 2137–2142. [CrossRef] [PubMed]
243. Kojima, T.; Watanabe, T.; Hata, K.; Shinozaki, M.; Yokoyama, T.; Nagawa, H. Cytomegalovirus Infection in Ulcerative Colitis. *Scand. J. Gastroenterol.* **2006**, *41*, 706–711. [CrossRef]
244. Yoshino, T.; Nakase, H.; Ueno, S.; Uza, N.; Inoue, S.; Mikami, S.; Matsuura, M.; Ohmori, K.; Sakurai, T.; Nagayama, S.; et al. Usefulness of Quantitative Real-Time PCR Assay for Early Detection of Cytomegalovirus Infection in Patients with Ulcerative Colitis Refractory to Immunosuppressive Therapies. *Inflamm. Bowel Dis.* **2007**, *13*, 1516–1521. [CrossRef]
245. Wada, Y.; Matsui, T.; Matake, H.; Sakurai, T.; Yamamoto, J.; Kikuchi, Y.; Yorioka, M.; Tsuda, S.; Yao, T.; Yao, S.; et al. Intractable Ulcerative Colitis Caused by Cytomegalovirus Infection: A Prospective Study on Prevalence, Diagnosis, and Treatment. *Dis. Colon. Rectum.* **2003**, *46*, S59-65. [CrossRef]
246. Minami, M.; Ohta, M.; Ohkura, T.; Ando, T.; Ohmiya, N.; Niwa, Y.; Goto, H. Cytomegalovirus Infection in Severe Ulcerative Colitis Patients Undergoing Continuous Intravenous Cyclosporine Treatment in Japan. *World J. Gastroenterol.* **2007**, *13*, 754–760. [CrossRef] [PubMed]
247. Kambham, N.; Vij, R.; Cartwright, C.A.; Longacre, T. Cytomegalovirus Infection in Steroid-Refractory Ulcerative Colitis: A Case-Control Study. *Am. J. Surg. Pathol.* **2004**, *28*, 365–373. [CrossRef] [PubMed]
248. Cottone, M.; Pietrosi, G.; Martorana, G.; Casà, A.; Pecoraro, G.; Oliva, L.; Orlando, A.; Rosselli, M.; Rizzo, A.; Pagliaro, L. Prevalence of Cytomegalovirus Infection in Severe Refractory Ulcerative and Crohn's Colitis. *Am. J. Gastroenterol.* **2001**, *96*, 773–775. [CrossRef] [PubMed]
249. Barahona-Garrido, J.; Martínez-Benítez, B.; Espinosa-Cárdenas, E.; Sarti, H.M.; Gutiérrez-Manjarrez, J.I.; Aguirre-Gutiérrez, R.; Tellez-Avila, F.I.; Coss-Adame, E.; García-Juárez, I.; Yamamoto-Furusho, J.K. Cytomegalovirus Infection in Patients Who Required Colectomy for Toxic Megacolon or Severe Steroid-Refractory Ulcerative Colitis. *Dig. Dis. Sci.* **2010**, *55*, 867–868. [CrossRef] [PubMed]
250. Maher, M.M.; Nassar, M.I. Acute Cytomegalovirus Infection Is a Risk Factor in Refractory and Complicated Inflammatory Bowel Disease. *Dig. Dis. Sci.* **2009**, *54*, 2456–2462. [CrossRef]
251. Dignass, A.; Lindsay, J.O.; Sturm, A.; Windsor, A.; Colombel, J.-F.; Allez, M.; D'Haens, G.; D'Hoore, A.; Mantzaris, G.; Novacek, G.; et al. Second European Evidence-Based Consensus on the Diagnosis and Management of Ulcerative Colitis Part 2: Current Management. *J. Crohns. Colitis* **2012**, *6*, 991–1030. [CrossRef] [PubMed]
252. Campos, S.T.; Portela, F.A.; Tomé, L. Cytomegalovirus, Inflammatory Bowel Disease, and Anti-TNFα. *Int. J. Colorectal. Dis.* **2017**, *32*, 645–650. [CrossRef]
253. Stein, J.; Volk, H.D.; Liebenthal, C.; Krüger, D.H.; Prösch, S. Tumour Necrosis Factor Alpha Stimulates the Activity of the Human Cytomegalovirus Major Immediate Early Enhancer/Promoter in Immature Monocytic Cells. *J. Gen. Virol.* **1993**, *74*, 2333–2338. [CrossRef]
254. Nakase, H.; Honzawa, Y.; Toyonaga, T.; Yamada, S.; Minami, N.; Yoshino, T.; Matsuura, M. Diagnosis and Treatment of Ulcerative Colitis with Cytomegalovirus Infection: Importance of Controlling Mucosal Inflammation to Prevent Cytomegalovirus Reactivation. *Intest. Res.* **2014**, *12*, 5–11. [CrossRef]

255. Matsumura, K.; Nakase, H.; Kosugi, I.; Honzawa, Y.; Yoshino, T.; Matsuura, M.; Kawasaki, H.; Arai, Y.; Iwashita, T.; Nagasawa, T.; et al. Establishment of a Novel Mouse Model of Ulcerative Colitis with Concomitant Cytomegalovirus Infection: In Vivo Identification of Cytomegalovirus Persistent Infected Cells. *Inflamm. Bowel Dis.* **2013**, *19*, 1951–1963. [CrossRef]
256. Chang, W.L.W.; Barry, P.A.; Szubin, R.; Wang, D.; Baumgarth, N. Human Cytomegalovirus Suppresses Type I Interferon Secretion by Plasmacytoid Dendritic Cells through Its Interleukin 10 Homolog. *Virology* **2009**, *390*, 330–337. [CrossRef]
257. Park, S.C.; Jeen, Y.M.; Jeen, Y.T. Approach to Cytomegalovirus Infections in Patients with Ulcerative Colitis. *Korean J. Intern. Med.* **2017**, *32*, 383–392. [CrossRef]
258. Fakhreddine, A.Y.; Frenette, C.T.; Konijeti, G.G. A Practical Review of Cytomegalovirus in Gastroenterology and Hepatology. *Gastroenterol. Res. Pract.* **2019**, *2019*, 6156581. [CrossRef]
259. Yerushalmy-Feler, A.; Padlipsky, J.; Cohen, S. Diagnosis and Management of CMV Colitis. *Curr. Infect. Dis. Rep.* **2019**, *21*, 5. [CrossRef] [PubMed]
260. Yokoyama, Y.; Yamakawa, T.; Hirano, T.; Kazama, T.; Hirayama, D.; Wagatsuma, K.; Nakase, H. Current Diagnostic and Therapeutic Approaches to Cytomegalovirus Infections in Ulcerative Colitis Patients Based on Clinical and Basic Research Data. *Int. J. Mol. Sci.* **2020**, *21*, 2438. [CrossRef] [PubMed]
261. Ciccocioppo, R.; Racca, F.; Paolucci, S.; Campanini, G.; Pozzi, L.; Betti, E.; Riboni, R.; Vanoli, A.; Baldanti, F.; Corazza, G.R. Human Cytomegalovirus and Epstein-Barr Virus Infection in Inflammatory Bowel Disease: Need for Mucosal Viral Load Measurement. *World J. Gastroenterol.* **2015**, *21*, 1915–1926. [CrossRef] [PubMed]
262. Ciccocioppo, R.; Racca, F.; Scudeller, L.; Piralla, A.; Formagnana, P.; Pozzi, L.; Betti, E.; Vanoli, A.; Riboni, R.; Kruzliak, P.; et al. Differential Cellular Localization of Epstein-Barr Virus and Human Cytomegalovirus in the Colonic Mucosa of Patients with Active or Quiescent Inflammatory Bowel Disease. *Immunol. Res.* **2016**, *64*, 191–203. [CrossRef]
263. Jones, A.; McCurdy, J.D.; Loftus, E.V.; Bruining, D.H.; Enders, F.T.; Killian, J.M.; Smyrk, T.C. Effects of Antiviral Therapy for Patients with Inflammatory Bowel Disease and a Positive Intestinal Biopsy for Cytomegalovirus. *Clin. Gastroenterol. Hepatol.* **2015**, *13*, 949–955. [CrossRef]
264. Beswick, L.; Ye, B.; van Langenberg, D.R. Toward an Algorithm for the Diagnosis and Management of CMV in Patients with Colitis. *Inflamm. Bowel Dis.* **2016**, *22*, 2966–2976. [CrossRef]
265. Shukla, T.; Singh, S.; Loftus, E.V.; Bruining, D.H.; McCurdy, J.D. Antiviral Therapy in Steroid-Refractory Ulcerative Colitis with Cytomegalovirus: Systematic Review and Meta-Analysis. *Inflamm. Bowel Dis.* **2015**, *21*, 2718–2725. [CrossRef]
266. Roblin, X.; Pillet, S.; Oussalah, A.; Berthelot, P.; Del Tedesco, E.; Phelip, J.-M.; Chambonnière, M.-L.; Garraud, O.; Peyrin-Biroulet, L.; Pozzetto, B. Cytomegalovirus Load in Inflamed Intestinal Tissue Is Predictive of Resistance to Immunosuppressive Therapy in Ulcerative Colitis. *Am. J. Gastroenterol.* **2011**, *106*, 2001–2008. [CrossRef]
267. Lévêque, N.; Brixi-Benmansour, H.; Reig, T.; Renois, F.; Talmud, D.; Brodard, V.; Coste, J.-F.; De Champs, C.; Andréoletti, L.; Diebold, M.-D. Low Frequency of Cytomegalovirus Infection during Exacerbations of Inflammatory Bowel Diseases. *J. Med. Virol.* **2010**, *82*, 1694–1700. [CrossRef]
268. Hommel, C.; Pillet, S.; Rahier, J.-F. Comment on: "Resolution of CMV Infection in the Bowel on Vedolizumab Therapy". *J. Crohns Colitis* **2020**, *14*, 148–149. [CrossRef] [PubMed]
269. Kopylov, U.; Papamichael, K.; Katsanos, K.; Waterman, M.; Bar-Gil Shitrit, A.; Boysen, T.; Portela, F.; Peixoto, A.; Szilagyi, A.; Silva, M.; et al. Impact of Infliximab and Cyclosporine on the Risk of Colectomy in Hospitalized Patients with Ulcerative Colitis Complicated by Cytomegalovirus-A Multicenter Retrospective Study. *Inflamm. Bowel Dis.* **2017**, *23*, 1605–1613. [CrossRef] [PubMed]
270. Sands, B.E.; Sandborn, W.J.; Panaccione, R.; O'Brien, C.D.; Zhang, H.; Johanns, J.; Adedokun, O.J.; Li, K.; Peyrin-Biroulet, L.; Van Assche, G.; et al. Ustekinumab as Induction and Maintenance Therapy for Ulcerative Colitis. *N. Engl. J. Med.* **2019**, *381*, 1201–1214. [CrossRef] [PubMed]
271. Henmi, Y.; Kakimoto, K.; Inoue, T.; Nakazawa, K.; Kubota, M.; Hara, A.; Mikami, T.; Naka, Y.; Hirata, Y.; Hirata, Y.; et al. Cytomegalovirus Infection in Ulcerative Colitis Assessed by Quantitative Polymerase Chain Reaction: Risk Factors and Effects of Immunosuppressants. *J. Clin. Biochem. Nutr.* **2018**, *63*, 246–251. [CrossRef] [PubMed]
272. Faulkner, C.L.; Luo, Y.X.; Isaacs, S.; Rawlinson, W.D.; Craig, M.E.; Kim, K.W. The Virome in Early Life and Childhood and Development of Islet Autoimmunity and Type 1 Diabetes: A Systematic Review and Meta-Analysis of Observational Studies. *Rev. Med. Virol.* **2020**, e2209. [CrossRef]
273. Mine, K.; Yoshikai, Y.; Takahashi, H.; Mori, H.; Anzai, K.; Nagafuchi, S. Genetic Susceptibility of the Host in Virus-Induced Diabetes. *Microorganisms* **2020**, *8*, 1133. [CrossRef]
274. Aarnisalo, J.; Veijola, R.; Vainionpää, R.; Simell, O.; Knip, M.; Ilonen, J. Cytomegalovirus Infection in Early Infancy: Risk of Induction and Progression of Autoimmunity Associated with Type 1 Diabetes. *Diabetologia* **2008**, *51*, 769–772. [CrossRef] [PubMed]
275. Hiltunen, M.; Hyöty, H.; Karjalainen, J.; Leinikki, P.; Knip, M.; Lounamaa, R.; Akerblom, H.K. Serological Evaluation of the Role of Cytomegalovirus in the Pathogenesis of IDDM: A Prospective Study. The Childhood Diabetes in Finland Study Group. *Diabetologia* **1995**, *38*, 705–710. [CrossRef] [PubMed]
276. Ivarsson, S.A.; Lindberg, B.; Nilsson, K.O.; Ahlfors, K.; Svanberg, L. The Prevalence of Type 1 Diabetes Mellitus at Follow-up of Swedish Infants Congenitally Infected with Cytomegalovirus. *Diabet Med.* **1993**, *10*, 521–523. [CrossRef]
277. Ward, K.P.; Galloway, W.H.; Auchterlonie, I.A. Congenital Cytomegalovirus Infection and Diabetes. *Lancet* **1979**, *1*, 497. [CrossRef]

278. Hyöoty, H.; Räasäanen, L.; Hiltunen, M.; Lehtinen, M.; Huupponen, T.; Leinikki, P. Decreased Antibody Reactivity to Epstein-Barr Virus Capsid Antigen in Type 1 (Insulin-Dependent) Diabetes Mellitus. *Apmis* **1991**, *99*, 359–363. [CrossRef]
279. Saber, A.Z.A.B.I.; Mohammed, A.H. The Roles of Human Cytomegalovirus and Epstein-Barr Virus in Type 1 Diabetes Mellitus. *Ann. Trop. Med. Health* **2019**, *22*, 90–99. [CrossRef]
280. Hjelmesæth, J.; Müller, F.; Jenssen, T.; Rollag, H.; Sagedal, S.; Hartmann, A. Is There a Link between Cytomegalovirus Infection and New-Onset Posttransplantation Diabetes Mellitus? Potential Mechanisms of Virus Induced β-Cell Damage. *Nephrol. Dial. Transplant.* **2005**, *20*, 2311–2315. [CrossRef]
281. Adland, E.; Klenerman, P.; Goulder, P.; Matthews, P.C. Ongoing Burden of Disease and Mortality from HIV/CMV Coinfection in Africa in the Antiretroviral Therapy Era. *Front. Microbiol.* **2015**, *6*, 1016. [CrossRef]
282. Clement, M.; Humphreys, I.R. Cytokine-Mediated Induction and Regulation of Tissue Damage During Cytomegalovirus Infection. *Front. Immunol.* **2019**, *10*, 78. [CrossRef]

Review

Where do we Stand after Decades of Studying Human Cytomegalovirus?

Francesca Gugliesi [1,†], Alessandra Coscia [2,†], Gloria Griffante [1], Ganna Galitska [1], Selina Pasquero [1], Camilla Albano [1] and Matteo Biolatti [1,*]

1 Laboratory of Pathogenesis of Viral Infections, Department of Public Health and Pediatric Sciences, University of Turin, 10126 Turin, Italy; francesca.gugliesi@unito.it (F.G.); gloria.griffante@unito.it (G.G.); ganna.galitska@unito.it (G.G.); selina.pasquero@unito.it (S.P.); camilla.albano@unito.it (C.A.)

2 Complex Structure Neonatology Unit, Department of Public Health and Pediatric Sciences, University of Turin, 10126 Turin, Italy; alessandra.coscia@unito.it

* Correspondence: matteo.biolatti@unito.it

† These authors contributed equally to this work.

Received: 19 March 2020; Accepted: 5 May 2020; Published: 8 May 2020

Abstract: Human cytomegalovirus (HCMV), a linear double-stranded DNA betaherpesvirus belonging to the family of Herpesviridae, is characterized by widespread seroprevalence, ranging between 56% and 94%, strictly dependent on the socioeconomic background of the country being considered. Typically, HCMV causes asymptomatic infection in the immunocompetent population, while in immunocompromised individuals or when transmitted vertically from the mother to the fetus it leads to systemic disease with severe complications and high mortality rate. Following primary infection, HCMV establishes a state of latency primarily in myeloid cells, from which it can be reactivated by various inflammatory stimuli. Several studies have shown that HCMV, despite being a DNA virus, is highly prone to genetic variability that strongly influences its replication and dissemination rates as well as cellular tropism. In this scenario, the few currently available drugs for the treatment of HCMV infections are characterized by high toxicity, poor oral bioavailability, and emerging resistance. Here, we review past and current literature that has greatly advanced our understanding of the biology and genetics of HCMV, stressing the urgent need for innovative and safe anti-HCMV therapies and effective vaccines to treat and prevent HCMV infections, particularly in vulnerable populations.

Keywords: human cytomegalovirus; genetic variability; viral dissemination; pathogenesis; antiviral therapy

---

1. Introduction

Human cytomegalovirus (HCMV), also called human herpesvirus 5 (HHV-5), is one of the nine herpesviruses capable of successfully infecting humans. HCMV belongs to the Group I of the Baltimore classification, and specifically to the subfamily *Betaherpesvirinae* within the *Herpesviridae* family (Table 1) [1].

Table 1. Classification of human herpesviruses.

Subfamily	Genus	Species	Tropism	Global Prevalence (%)
Alphaherpesvirinae	Simplexvirus	Human herpesvirus 1 (HHV-1)/Herpes simplex virus type 1 (HSV-1)	Mucoepithelial cells (mainly oro-facial tract), neurons	40–90
		Human herpesvirus 2 (HHV-2)/Herpes simplex virus type 2 (HSV-2)	Mucoepithelial cells (mainly genital tract), neurons	10–60
	Varicellovirus	Human herpesvirus 3 (HHV-3)/Varicella zoster virus (VZV)	Mucoepithelial cells, T cells, neurons	50–95
Betaherpesvirinae	Cytomegalovirus	Human herpesvirus 5 (HHV-5)/Human cytomegalovirus (HCMV)	Epithelial cells, monocytes, lymphocytes, fibroblasts, and more	56–94
	Roseolovirus	Human herpesvirus 6A (HHV-6A)	Epithelial cells, T cells, fibroblasts	60–100
		Human herpesvirus 6B (HHV-6B)	Epithelial cells, T cells, fibroblasts	40–100
		Human herpesvirus 7 (HHV-7)	Epithelial cells, T cells, fibroblasts	44–98
Gammaherpesvirinae	Lymphocryptovirus	Human herpesvirus 4 (HHV-4)/Epstein-Barr virus (EBV)	Mucoepithelial cells, B cells	80–100
	Rhadinovirus	Human herpesvirus 8 (HHV-8)/Kaposi's sarcoma associated herpesvirus (KSHV)	Lymphocytes	6–50

The expression of HCMV genes, similar to that of all other herpesviruses, occurs in a temporal cascade consisting of immediate-early (IE), early (E), and late (L) genes. The viral particles are formed by a double-stranded DNA (dsDNA) genome (~230 kb), an icosahedral capsid, followed by the tegument (a proteinaceous layer), and a coating known as pericapsid or envelope, which confers the virion a quasi-spherical shape (Figure 1), a feature shared with all other herpesviruses.

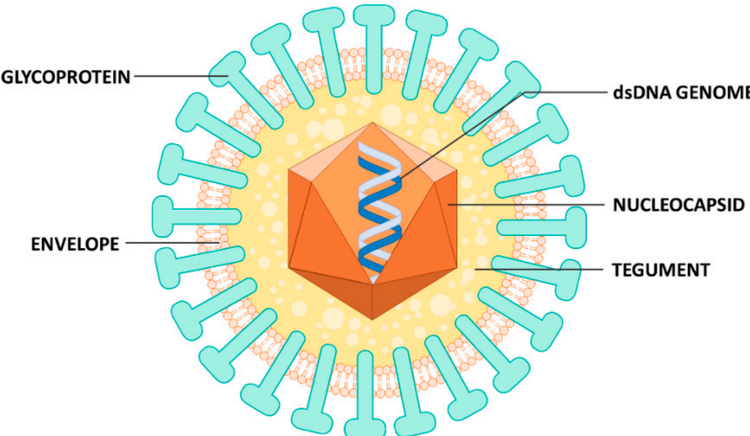


Figure 1. Structure of HCMV virion. Mature virions are coated by an envelope, from which viral glycoproteins protrude, and contain a double-stranded DNA genome enclosed within an icosahedral symmetry capsid, that is surrounded by tegument.

HCMV can infect a broad cell range that includes epithelial cells of glandular and mucous tissues, smooth muscle cells, fibroblasts, macrophages, hepatocytes, dendritic cells, and vascular endothelial cells (ECs) [2]. After primary infection, similar to other members of the herpesvirus family, HCMV can establish latency in the host that can be reversed even after many years by any number of stimuli [3,4].

A typical characteristic of HCMV, which originally granted the virus its name, is that of forming in the infected cell a voluminous intranuclear inclusion body and one or more intra-cytoplasmic inclusion bodies, the so-called "owl's eye" inclusions, made up of clusters of newly formed viruses and lysosomes. The formation of such bodies generally results in increased cellular volume, a phenomenon defined as cytomegaly. Studies on HCMV began in the early 1900s when particular attention was paid to the owl's eyes found in biopsies from stillborn fetuses and later in the kidneys and parathyroid gland cells of organ transplant patients [4].

HCMV efficiently spreads through infected body fluids, and it can also be transmitted vertically from the mother to the fetus through the placenta, causing congenital pathologies. Furthermore, even when the primary infection is resolved by an effective cellular immune response, a population of latently infected myeloid cells can persist in bone marrow monocyte precursors, thereby contributing to the risk of transferring HCMV along with organs and tissues following transplantation.

HCMV is a common pathogen of global clinical relevance, with worldwide seroprevalence ranging from 56% to 94% [5]. The viral spread in the global population is enormous, mainly due to the asymptomatic mode of infection, followed by a constant shedding of the virus through body fluids (e.g., milk, saliva, cervical secretions, and tears), which can last for months or even years.

HCMV is particularly dangerous for the following target categories of individuals [6]: (i) immunocompetent hosts, where it causes asymptomatic infections or a slight form of mononucleotic-like pathology; (ii) immunocompromised individuals such as patients suffering from human immunodeficiency virus (HIV) or undergoing bone or organ transplants; and (iii) congenitally infected newborns, who can be infected in utero, postnatally, or via breastfeeding. Of note, the prevalence of congenital HCMV (cHCMV) disease is much higher than that of Down syndrome, spina bifida, or fetal alcohol syndrome [7].

The host immune status ultimately determines the outcome of the infection as immunocompromised conditions predispose the patient to a primary infection or determine the reactivation of a latent one. In this regard, HCMV is notoriously famous for its ability to cause congenital anomalies and long-term neurological sequelae in newborns. Furthermore, it can also trigger the development of serious pathologies in solid organ or stem cell transplant recipients that are not always resolved by currently available antivirals, thereby leading in some cases to death [8].

This review provides a summary of the general characteristics of HCMV as well as its strain variability, dissemination, latency, reactivation, pathogenesis, prevention, and treatment.

2. Pathogenesis

HCMV pathogenesis and clinical features of infection in various patient populations are summarized below (Figure 2).

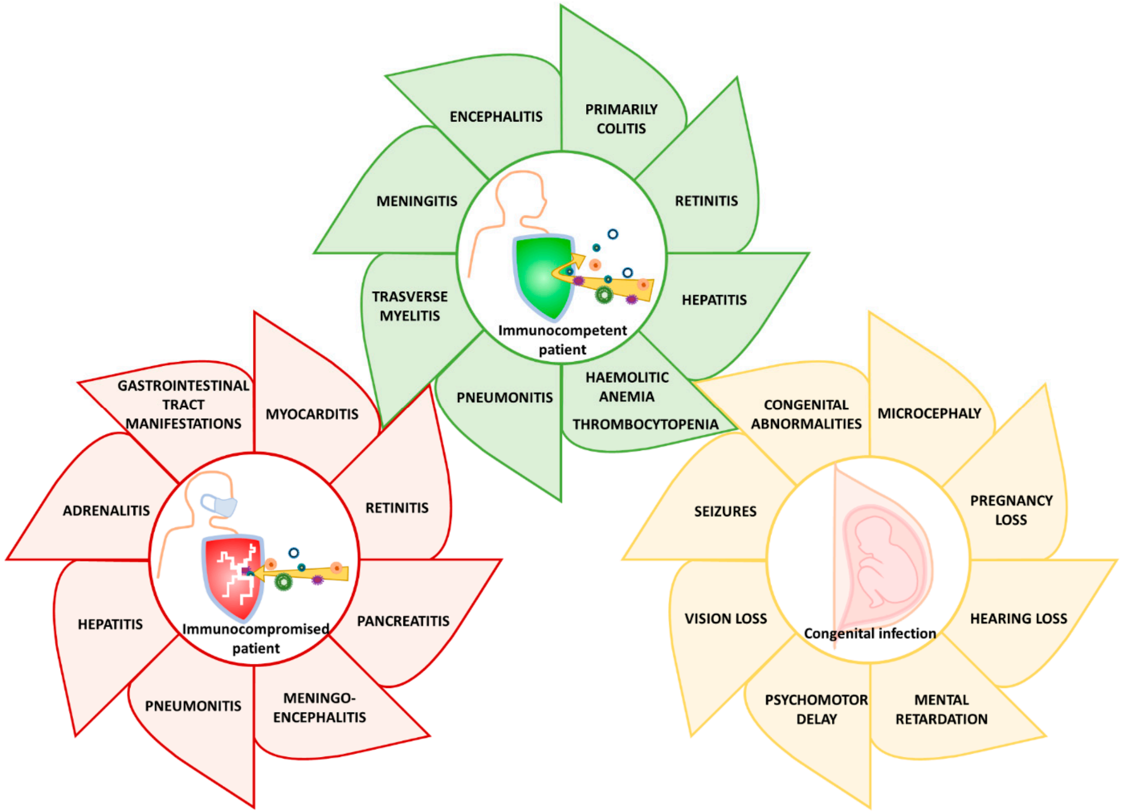


Figure 2. HCMV clinical manifestations in immunocompetent individuals with severe HCMV infection, in immunocompromised people, especially in acquired immune deficiency syndrome (AIDS) patients, transplant recipients, and upon congenital infection.

2.1. *Infection of Immunocompetent Adults*

HCMV infection commonly occurs in healthy adults and children, with a prevalence gradually increasing with age [9]. When symptomatic, it results in a mononucleosis-like syndrome with a less prominent cervical lymphadenopathy than that caused by the Epstein-Barr virus (EBV) [10]. One of the symptoms is rash, which only manifests in 30% of HCMV mononucleosis cases [11]. Noteworthy, a minority of primary HCMV infections result in relapsing symptoms (i.e., fever, night sweats, fatigue, myalgia, arthralgia, and transaminitis), which can last for several weeks [10] or, less frequently, lead to multi-organ failure [12,13], even though the severe tissue-invasive disease is usually limited to critically ill or immunodeficient patients [14]. A study describing the various clinical manifestations of 290 immunocompetent patients with severe HCMV infection showed that the gastrointestinal tract is the preferential organ affected, primarily in the form of colitis, followed by morbidities of the central nervous system (CNS) (i.e., meningitis, encephalitis, and transverse myelitis), hematological abnormalities (i.e., hemolytic anemia, and thrombocytopenia), the involvement of the eye (uveitis and retinitis), liver (hepatitis), and lung (pneumonitis), and thrombosis of the arterial and venous system [15]. Although several studies have reported a rapid clinical improvement in immunocompetent patients with severe HCMV infection after anti-HCMV therapy, criteria for specific antiviral pharmacological treatments are not well established [12]. Randomized controlled trials should therefore be conducted to determine in which cases anti-HCMV therapy in immunocompetent patients with symptomatic HCMV infections is needed.

2.2. *Infection of Immunocompromised Patients*

In the immunocompetent host, HCMV and immunity coexist in a delicate balance. When the host immune system is compromised—i.e., in individuals with acquired immune deficiency syndrome

(AIDS) and other immune diseases, post-transplant and intensive care unit (ICU) patients, and, to some extent, elderly people—the virus can exert its full pathogenic potential. Its reactivation in immunocompetent hosts, which occurs intermittently throughout life, triggers a lifelong IgG-mediated immunologic response that keeps in check viral replication. In contrast, uncontrolled viral replication occurs when populations of HCMV-specific $CD4^+$ and $CD8^+$ T cells are not well preserved, as observed in immunocompromised hosts, leading to severe clinical disease [16].

2.3. Cytomegalovirus and HIV

HIV-infected individuals are generally co-infected with HCMV [17]. Prior to the introduction of highly active antiretroviral therapy (HAART) in developed countries, about 40% of HIV-infected patients would suffer from severe HCMV disease [18]. Currently, durable suppression of HIV viremia has increased the overall patient life quality and expectancy and reduced to a minimum the pathologies associated with HCMV viral reactivation. Nevertheless, comorbidities remain problematic for HIV patients. In this regard, a close relationship between HCMV infection and HIV persistence has been reported. This is probably due to the fact that HIV-driven $CD4^+$ T-cell loss and dysfunction may lead to HCMV replication and subsequent expansion of $CD8^+$ T cells. Indeed, an elevated number of $CD8^+$ T cells and a low $CD4^+/CD8^+$ T-cell ratio have been observed in individuals co-infected with both viruses but not in patients infected with HIV or HCMV alone [19]. Fittingly, Hunt et al. [20] demonstrated that CD8 T-cell activation could be reduced in HAART-treated HIV patients with incomplete CD4 T-cell recovery by administering anti-HCMV drugs, attesting that HCMV plays a significant role in immune activation in HIV patients. Moreover, persistent HCMV replication modulated longevity and proliferation of HIV-infected cells, improved the recruitment of new HIV target cells, and stimulated HIV transcription, thereby creating an HIV reservoir favoring AIDS progression. Clinically, retinitis has been shown to be the predominant pathology in AIDS patients (20–30%), and it usually appears at the late stages of the syndrome in patients with low CD4 count [21]. HCMV retinitis in HIV patients is commonly observed in two different forms: fulminant or indolent, both characterized by minimal or completely absent vitreous and anterior chamber inflammation [22]. If left untreated, HCMV infection of retinal cells may cause subacute retinal destruction, which can result in irreversible blindness. Paradoxically, HAART therapy while restoring the patient immune system can lead to a new pathology, known as immune recovery uveitis (IRU), which is equally destructive to the host tissue and deleterious to the quality of the patient's life [23]. Following retinitis, the most prevalent clinical manifestations are the following: colitis(in the US, 5–10% of HIV patients with low CD4 lymphocyte counts were affected by enterocolitis prior to the availability of HAART therapy [24]), esophagitis (most commonly due to co-infection with either herpes simplex virus or *Candida albicans*), pneumonitis, encephalitis, hepatitis, and adrenalitis [25].

2.4. Cytomegalovirus and Transplant Patients

HCMV is one of the most frequently encountered opportunistic viral pathogens in transplant patients: a primary infection can occur in seronegative individuals after organ transplantation while a latent infection can be reactivated in seropositive individuals due to immunosuppressive treatment. The risks of HCMV-related complications in transplant recipients (R) vary according to the serostatus of the donor (D): HCMV D^-/R^- transplantation is classified as low-risk, HCMV D^+/R^+ or D^-/R^+ as medium-risk, and HCMV D^+/R^- as high-risk [26,27]. The most prevalent clinical manifestations in HCMV-transplanted patients are gastrointestinal symptoms, mainly affecting the upper digestive tract, whereas diarrhea is a rare occurrence indicative of colon involvement. Meningoencephalitis, clinical hepatitis, myocarditis, and pancreatitis are more common than respiratory symptoms, which in fact indicate more severe disease and may require admission to an ICU. Transplant patients without HCMV prophylaxis may display a spectrum of clinical manifestations that vary in severity from patient to patient, depending on additional personal illness risk factors, type of transplant procedure, the immunological match between donor and recipient, and immunosuppressive drugs being

administered. For instance, patients treated with mammalian target of rapamycin (mTOR) inhibitors display a very low incidence of HCMV. The incidence of HCMV also correlates with the type of transplant: about 50% among pancreas or kidney–pancreas recipients, 50–75% in lung or heart–lung recipients, 9–23% in heart recipients, 22–29% in liver recipients, and 8–32% in kidney recipients [28,29]. Moreover, in patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT), HCMV can lead to fatal infectious complications related to host immune recovery—about 30–70% of non-autologous and 5% of autologous HSCT-patients develop HCMV disease. Pneumonia is the disease most highly associated with HCMV infection of HSCT patients, frequently leading to death despite aggressive treatment with antiviral agents and adjunctive therapies [30]. The reasons for the development of different clinical sequelae among all the aforementioned types of transplanted patients is probably due to a combination of the following factors: (i) the nature of the proinflammatory cytokine cocktail arising after organ transplantation; (ii) the duration of HCMV replication—most transplant recipients display acute HCMV infection, which subsequently results in disease in a relatively short time frame, whereas, for example, congenitally infected infants and HIV patients can display high levels of HCMV replication for several months; and (iii) the status of the immune system response. For instance, HCMV pneumonitis only occurs when patients can activate their immune system [31].

Interestingly, conflicting results have been reported regarding the association between early cytomegalovirus reactivation and relapse after HSCT. Some studies suggest that HCMV replication after transplantation is associated with a decreased relapse risk [32–37], while others highlight that HCMV's protective effect is restricted to patients with acute myeloid leukemia (AML) [38,39] and cannot be extended to patients with acute lymphoblastic leukemia (ALL) [40], lymphoma [41], myelodysplastic syndrome (MDS) [42], or observed in pediatric leukemias [43]. Furthermore, a more recent study by Peric et al. [44] reports that the protective HCMV effect has been pronounced in patients with myeloproliferative malignancies, while, at the same time, confirming the fact that such effect has not been observed in patients with lymphoproliferative disorders, in concordance with other studies [40,41]. Moreover, the same study highlights a significant reduction of relapse in patients with myeloproliferative neoplasms (MPN) associated with early CMV reactivation [44]. Finally, some evidence suggests that the beneficial effect of HCMV is mostly related to the conditioning regimen and restricted only to patients who receive myeloablative chemotherapy (MAC) before undergoing HSCT [45]. Despite the mounting evidence, the impact of HCMV reactivation on the patients' overall survival (OS) has been largely regarded as controversial due to its known negative effect on non-relapse mortality (NRM). Thus, it remains largely unclear whether these conflicting reports can provide a more detailed insight into the distinct protective mechanism or they simply reflect other variables, including the sample size of the studied transplant groups. Therefore, larger prospective studies with a significant follow-up on all patients with different malignancies, monitored and treated in a homogeneous manner are needed to fully elucidate the underlying mechanism responsible for the exact effect of HCMV reactivation. These findings may ultimately lead to a significant improvement in patient management, donor selection strategies, or more personalized preemptive treatment of HCMV infection in posttransplant patients with particular malignancies.

2.5. Congenital and Neonatal Infection

HCMV is the major infectious cause of congenital abnormalities. The incidence of cHCMV infection due to primary and non-primary maternal HCMV infection is ~0.4–0.8% in developed countries. In general, the risk of transmission correlated with the stage of pregnancy is higher in later stages and lower in earlier ones, but in either case HCMV infection is generally associated with severe clinical sequelae in the fetus. In developed countries, ~40% of women in reproductive age are HCMV seronegative, 1–3% of whom may contract primary HCMV infection during pregnancy. The most vulnerable groups include adolescents, mothers, and caregivers in close contact with young children (e.g., teachers, nurses, etc.). Primary maternal HCMV infection has a 30–40% risk of transmission to the

fetus. Importantly, HCMV reactivation or reinfection can also occur in women who are seropositive prior to pregnancy, but, in such cases, the rate of HCMV transmission is only ~1% [46,47].

The hypothesis that pre-existing maternal immunity may favor low HCMV transmission rates has long been debated. In this regard, Coppola et al. [48] performed a systemic review of the literature using Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA) [49] guidelines and identified 19 studies assessing congenital HCMV birth prevalence in HCMV-seropositive mothers. All these studies reported low levels of congenital HCMV birth prevalence (0.4–0.6%) in seropositive mothers, in good agreement with previous findings by Lanzieri et al. [50], who systematically reviewed postnatal HCMV prevalence in developing countries. Moreover, 11 studies reported HCMV maternal seroprevalence and HCMV birth prevalence rates of 84–100% and 0.6–6.1%, respectively.

Even though most infants with cHCMV are asymptomatic, they may develop health problems at birth or later. In the most severe cases, cHCMV can cause the death of the unborn baby. In all other cases, the most clinically relevant signs at birth may include microcephaly, hepatosplenomegaly, retinitis, intrauterine growth restriction, seizures, rash, and jaundice. Some children with cHCMV infection may also suffer from long-term health problems, such as hearing loss, which can be present at birth or may develop later even in asymptomatic infants, developmental and motor delay, vision loss, and seizures. Cognitive impairment and retinitis have also been observed in asymptomatic children but at much lower rates compared to symptomatic children [51]. Prevention of HCMV remains elusive given the lack of drugs capable of treating HCMV infection in pregnant women. This aspect, together with the fact that maternal immunity has a protective role against cHCMV infection, suggests that vaccine development remains the most viable option to avoid HCMV vertical transmission.

3. Dissemination

HCMV exploits both vertical and horizontal transmission. Vertical transmission occurs through the placenta [52–54], during birth (with genital secretions), or postnatally through breast milk [55–57]. Horizontal transmission takes place via organ transplant [58,59], blood transfusion, or direct contact with contaminated body fluids, such as urine, breast milk, and genital secretions [60–62].

In most of these cases, because they cover all body surfaces, epithelial cells of the skin and internal mucosa are the first site of HCMV infection. For instance, infection through breastfeeding starts from the oral mucosa, moves to the gastrointestinal tract, which can support a productive infection, and eventually disseminates throughout the body. In contrast, studies using murine CMV (MCMV) have shown that, after oral/intranasal inoculation, the infection can only evolve in the upper respiratory tract but not in the gut [63]. It remains a matter of debate how HCMV disseminates from the upper respiratory tract throughout the body.

It is widely acknowledged that HCMV can spread systemically via leucocytes, a process associated with short-duration viremia, during which infection of the lungs, liver, and spleen occurs primarily through viral dissemination [64]. Subsequent secondary dissemination leads to the infection of salivary glands, breast, and kidneys, all secretion-producing organs that release the virus into the environment for months, even years, favoring intra-host transmission [64]. According to a model whereby primary dissemination produces many viral particles that then infect other organs generating even more virus progeny, it would be expected a gradual increase in viral burden during primary infection [64] (Figure 3).

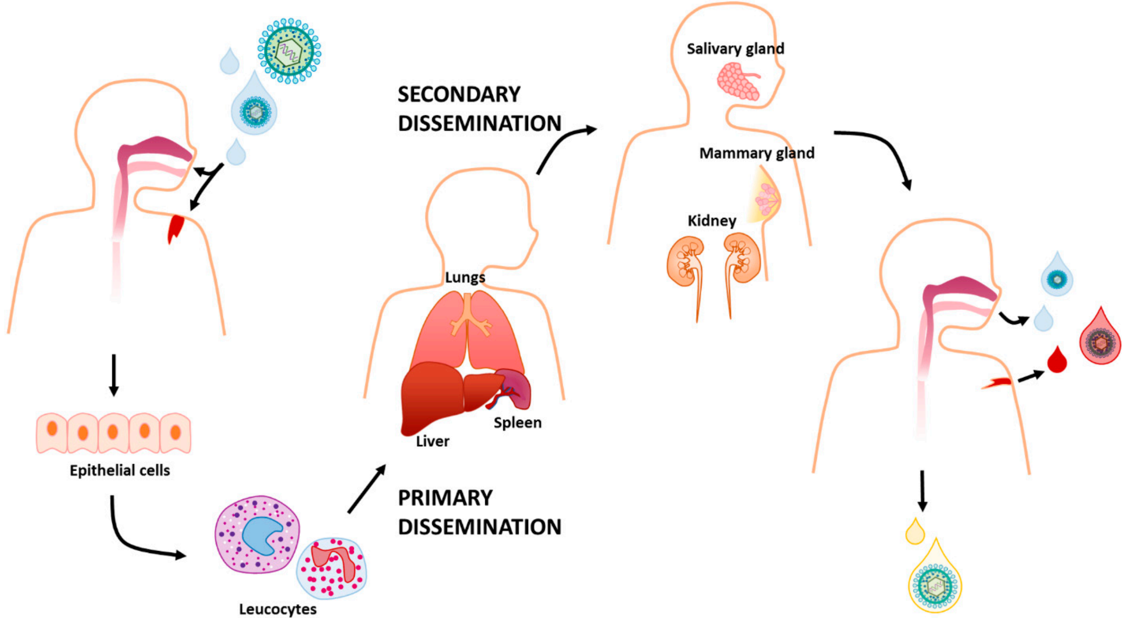


Figure 3. HCMV can be transmitted directly from person to person through bodily fluids including saliva, urine, cervical, and vaginal secretions, breast milk, semen, blood, and tears. It infects a new host usually by getting in through the upper gastrointestinal gastrointestinal tract or the respiratory tract. Here, the epithelial cells are often the first site of infection and from there HCMV infects leucocytes that traffic around the body. This is correlated with a process called primary viral dissemination that leads to the infection of multiple tissues, such as lungs, liver, and spleen. Afterwards, secondary viral dissemination spreads the infection to secretion-producing organs, such as salivary and mammary glands and kidneys, which shed the virus.

However, animal studies using the MCMV model showed a biphasic rather than a gradual increase in viremia, suggesting a much more complex scenario [65].

Recently, Jackson and Sparer [66] demonstrated that cells of the upper respiratory tract, once infected, release not only viral progeny but also chemotactic factors. According to the proposed model, these chemokines trigger the recruitment of innate immune cells, which after being infected further spread the virus to secondary organs and body fluids [66]. Fittingly, HCMV DNA has never been found in the form of free circulating viral particles, except for highly fragmented DNA [67]. Consistent with the lack of free circulating HCMV, leukocyte-depleted blood from seropositive donors prior to blood transfusion prevents HCMV transfer [68,69], indicating that HCMV viremia is mostly cell-associated. More recently, Farrell et al. [63] showed that the first cells to be infected after nasal inoculation with MCMV are alveolar macrophages and type 2 alveolar epithelial cells. Entry into epithelial cells and macrophages occurs through endocytosis and is followed by subsequent pH-dependent fusion with the endosomal membrane, mediated by the viral envelope glycoproteins gB and gH/gL/gO and the pentameric complex formed by gH/gL/UL128, UL130 and UL131A [70,71]. Then, the local spread is thought to occur through direct cell-to-cell transmission, mediated in part by the HCMV gene *US28* [72].

The main cell types contributing to hematogenous dissemination, albeit to different extents, include polymorphonuclear cells (PMNs), monocytes, ECs, and dendritic cells. After recruitment to the first site of infection, these cells are highly prone to infection themselves, thereby becoming potential vehicles for HCMV transmission, even though most of them are unable to support a complete viral replication cycle [73–76]. Consistently, HCMV is frequently found in PMNs from immunocompromised patients [74], in which viral replication is generally abortive and non-productive [73]. The infection of PMNs most likely occurs by transient microfusion between ECs and PMNs after an initial direct contact mediated by the pentameric complex. Successively, infected PMNs transfer the virus particles

to other cell types [77]. On the other hand, other studies using MCMV do not seem to support the hypothesis that neutrophils play a role in HCMV dissemination, since their depletion did not alter primary or secondary viral diffusion [78], whereas depletion of monocytes, macrophages, and NK led to reduced viral dissemination [63,79,80]. However, it is important to point out that there are substantial differences between human and murine CMV, exemplified by the lack of the MCMV CXC chemokine homolog involved in neutrophil migration [78].

HCMV carries two genes, *UL146* and *UL147*, which encode for the two chemokine homologs vCXCL-1 and vCXCL-2, respectively, involved in the recruitment of innate immune cells [81–84]. pUL128, a key component of the pentameric complex, is an important chemokine that, once released in the extracellular milieu, regulates monocyte migration [85]. Likewise, MCK2 in MCMV acts as a strong attractant of monocytes, which appear to be conserved dissemination vehicles across species [86,87]. Monocyte-driven hematogenous spread is probably the result of the close proximity of these cells to the vascular epithelium, which renders them particularly susceptible to infection with viral particles originating from productively infected ECs. Once fully differentiated into tissue macrophages [75], they can, in turn, spread the infection to the organs where they transmigrated [88–90].

Infected ECs also play a fundamental and active role in HCMV dissemination. In fact, HCMV infection of ECs supports viral replication and promotes the enhanced expression of the adhesion molecules ICAM-1 and vCAM-1 [91,92], as well as increased vascular permeability, which promotes recruitment of leucocytes, direct contact [92] and migration through the endothelial layer.

Dendritic cells (DCs) are antigen-presenting cells that keep in check foreign pathogens by influencing T-cell activation and differentiation in the draining lymph node through different mechanisms [93]. Immature DCs localize in all mucosal and epidermal surfaces of the body where they uptake HCMV infectious particles, thereby initiating the maturation process during their migration to the draining lymph node. Upon localization in this new site, the newly mature and permissive DCs are capable of transferring the virus to other cells [94].

In summary, there is still certainly a long way to go before we can fully understand the pathogenesis of HCMV infection, but the aforementioned mechanism of HCMV dissemination proposed by Jackson and Sparer [66] appears to be putting together many pieces of the puzzle.

4. Latency

Viral latency is defined as the maintenance of the viral genome without any production of infectious progeny until this dormant genome can reactivate in response to specific stimuli and initiate a productive infection. It is, therefore, becoming increasingly clear that a better understanding of latency and subsequent reactivation may be crucial to elucidate HCMV pathogenesis and develop therapeutics targeting latent virus reservoirs. This is a particularly important aspect given that all commercially available drugs for the treatment of HCMV diseases only target lytic but not latent infections.

For decades, latency was considered as a silent state of the infection, characterized by overall suppression of viral gene expression aimed at preventing the detection and activation of the immune system. However, several recent studies have shown latency to be a dynamic phase of the infection, where viral gene expression triggers a transcriptional cascade responsible for subverting host cell functions, such as cell survival, genome carriage, and immune evasion [95–97].

The main site where HCMV is known to establish latency is in cells of the myeloid lineage. The idea that infectious viral particles could be carried by white blood cells came from the observation that blood transfer from healthy seropositive donors to immunosuppressed seronegative recipients often resulted in HCMV disease [98–100], and that transfusion of leukocyte-depleted blood reduced the incidence of HCMV disease [101].

However, it was only thanks to the increased sensitivity of the PCR technique that HCMV DNA could be found in naturally latently infected peripheral blood mononuclear cells (PBMCs), in particular monocytes and $CD34^+$ progenitor cells isolated from the bone marrow [102,103]. Consistent with the

notion of myeloid cells being a bona fide site of latency, HCMV IE RNA expression has also recently been detected in DCs isolated from peripheral blood of healthy individuals [104].

Investigations on viral gene expression during natural infection are limited by the fact that only 0.004–0.01% of mononuclear cells from seropositive granulocyte colony-stimulating factor (G-CSF)-stimulated donors carry viral genomes, with a low copy number of 2–13 genomes per infected cell, as judged by PCR-driven in situ hybridization [105]. For this and other reasons, leukemic cell line models such as THP-1 and CD34$^+$ Kasumi 3, as well as several embryonic stem cell lines, have been preferentially used as bona fide and low-cost models to study latency and reactivation in vitro [106–109].

As CD34$^+$ cells are also lymphoid cell progenitors, several studies have tried to explain why myeloid cells are then the only cell lineage able to carry latent viral genome. By performing experimentally latent HCMV infection of CD34$^+$ progenitor cells, Poole et al. [110] observed an increase in the cellular transcription factor GATA-2, a key regulator of myeloid differentiation, suggesting that the virus may only engage myeloid-committed cells, promoting their survival. GATA-2 is also involved in the differentiation of hematopoietic progenitors along the endothelial lineage. However, HCMV DNA could not be detected by PCR in ECs from the saphenous vein of healthy individuals, contrary to the hypothesis that microvasculature is a site of HCMV latency [111].

To establish latency, HCMV must stop the production of infectious viral particles through suppression of viral gene expression and, at the same time, induce the expression of latency-associated viral genes [112–116]. One of the key events required to initiate a state of latency involves the repression of the viral major immediate-early promoter (MIEP), which is sufficient to prevent the expression of E and late L genes as well. Transcriptional inactivation of this region is achieved through induction of repressive chromatin marks—e.g., histones methylation and recruitment of heterochromatin protein 1 (HP-1)—and repressive transcription factors [117]. Concurrently, differentiation of latent CD34$^+$ cells or monocytes to macrophages or DCs induces re-activation of the promoter through histone acetylation and loss of HP-1, with subsequent expression of *IE* genes and re-entry into the lytic cycle [76,104,118,119], indicating that dynamic regulation of the MIEP is a first and crucial step to control latency/reactivation.

One of the most widely accepted hypotheses is that the virus gene expression upon latency is mainly characterized by a robust suppression and shut down of almost all viral genes, an expression profile similar to that of the late lytic cycle. In this regard, it has been proposed that, in latently infected cells, the timely transcriptional cascade of productive infection may be prematurely interrupted by cellular mechanisms. Alternatively, there could be, right after viral entry, early induction of viral gene expression followed by massive repression of viral transcription [120].

As mentioned above, rather than being quiescent, latent HCMV infection induces the expression of a certain amount of viral genes. The most sophisticated mechanism for modulating the host cell environment without attracting an immune response is mediated by non-immunogenic molecules, such as small RNA transcripts. Assessing both experimentally and naturally latent infected cells by next-generation sequencing, Rossetto et al. [121] identified two long non-coding (nc) RNAs (lncRNAs), RNA4.9 and RNA2.7, and mRNAs encoding replication factors UL84 and UL44. Of note, RNA lnc4.9 in concert with latently expressed *UL84* was shown to interact with members of the polycomb repressor complex 2 (PRC2), which potentially represents an additional step of silencing of the MIEP through their histone methyltransferase activity [122].

Across its genome, HCMV also encodes at least 20 viral microRNAs (miRNAs) identified first in lytically-infected cells [123], but also in latently-infected cells THP-1 by Meshesha et al. [124], using deep-sequencing analysis. More recently, two similar studies were performed using instead primary latently-infected cells that more resemble the in vivo situation, even though they showed conflicting results to some extent [125,126]. The advantage of using miRNAs, besides their non-immunogenic state, stems from their ability to modulate the expression of multiple targets involved in immune evasion, survival, and proliferation of HCMV-infected cells, as well as virus

reactivation [127]. One example is the miR-UL148D that during the lytic cycle promotes T-cell chemotaxis by targeting CCL5 (RANTES), while during latency it may trigger activin signaling, thereby inhibiting pro-inflammatory cytokine secretion [126,128]. In addition, even cellular miRNAome was shown to be widely affected by HCMV latent infection [129].

A restricted amount of viral proteins is detected in naturally latent infected cells, even though their exact role in latency is only partially clear. These include: US28, a constitutively activated chemokine receptor acting as a chemokine sink [130]; viral IL-10, which can downregulate MHC II surface expression and modulate CD4$^+$ T-cell recognition [131], UL144, a decoy tumor necrosis factor receptor (TNFR), which inhibits T cell proliferation in vitro [132] and subverts the TH1 immune response in a TNF ligand-independent fashion [133]; LUNA (latency unique natural antigen), a protein required for reactivation [134]; and UL138, which maintains latent infection and suppresses reactivation [115]. Interestingly, this latter is a potentially druggable target in latently infected cells as it inhibits a cellular drug transporter [134].

As latency appears to be a very complex phenomenon, reactivation is often the result of a closely intertwined crosstalk between cellular and viral signals triggering multiple pathways. Indeed, *IE* gene expression alone does not seem to be sufficient to induce the production of infectious particles [76]. In this regard, the observation that differentiation of experimentally latently infected monocytes into monocyte-derived macrophages can lead to a fully permissive phenotype [88,89] implies that the differentiation status is a critical determinant of reactivation. In addition, mounting evidence indicates that inflammation may also play a role in HCMV reactivation. For instance, virus reactivation has been observed in several progenitor cell types under a variety of inflammatory conditions [135–137], and HCMV disease prevalence is directly associated with highly inflammatory environments [137–139].

In light of the above, it is becoming increasingly evident how a multiplicity of latency and reactivation pathways can determine the course of HCMV infection. These pathways appear to be independent of the clinical strains but seemingly dependent on a combination of viral and cellular factors working cooperatively to cross the threshold for reactivation of latently infected cells (Figure 4).

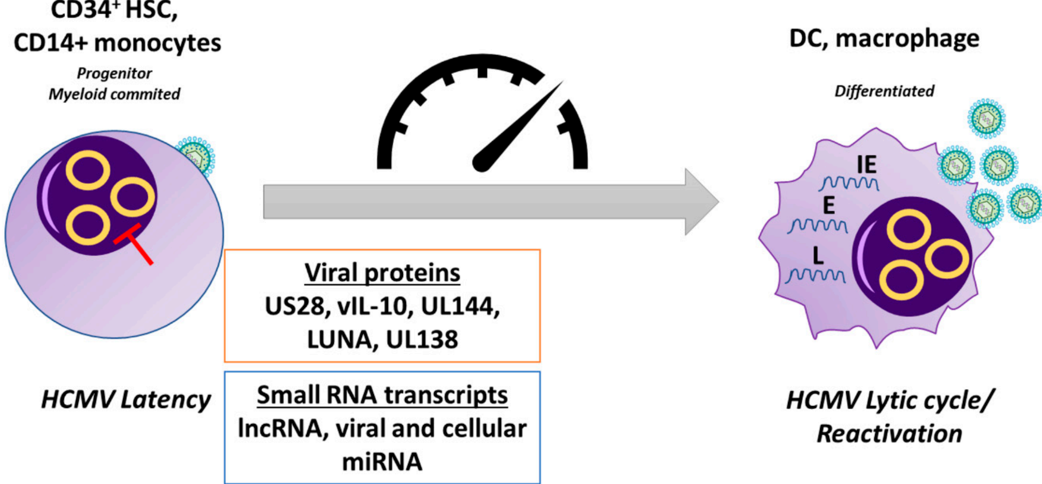


Figure 4. Latency. Following primary infection, HCMV can establish latency in CD34$^+$ myeloid progenitor cells and is carried down the myeloid lineage. In latently-infected CD34$^+$ cells and monocytes, there is a targeted suppression of lytic viral gene expression. HCMV utilizes several viral proteins and small RNA transcripts, including viral and cellular miRNAs, during latent infection to alter the signaling environment within the cell to maintain the status of latency. Differentiation of these cells to macrophages and DCs causes the derepression of the MIEP and allows initiation of the lytic transcription program, which involves a temporal cascade of viral gene transcription, allowing reactivation of de novo virus production. HCMV, human cytomegalovirus; DC, dendritic cell; HSC, hematopoietic stem cell; lncRNA, long non-coding RNA; IE, immediate-early; E, early; L, late.

5. HCMV Strain Variation

The massive spread of HCMV infection and the wide spectrum of disease manifestations in infected patients sparked a major interest in determining the origin and mechanisms of HCMV pathogenicity. It has been 66 years since Margaret Gladys Smith isolated for the first time MCMV from salivary glands and propagated the virus in mouse cell culture—such strain is still in use and commonly called "Smith strain" [140]. One year later, she succeeded in growing submaxillary salivary-derived HCMV in human cell culture. As early as 1960, Thomas Weller isolated the so-called David strain from a liver biopsy and managed to identify serological differences between cytomegalic inclusion disease (CID) isolates [141].. Subsequently, five different HCMV clinical isolates were sequenced (FIX GenBank AC146907; TR GenBank KF021605; PH GenBank AC146904; Toledo GenBank AC146905; Merlin GenBank AY446894). The pioneering work on sequencing of the complete genome of an HCMV laboratory strain (AD169 GenBank AC146999) [142], along with numerous in vitro findings and data from several vaccine studies, revealed the existence of a substantial genetic variation among HCMV strains. In particular, the highly passaged laboratory strains AD169 and Towne (GenBank FJ616285) appeared attenuated when administered as vaccine candidates [143,144]. By contrast, the Toledo strain, which had only been passaged several times in culture, caused disease when administered to seropositive individuals [145]. Taken together, these findings suggest that the pathogenic potential of HCMV was correlated with the genetic composition of each distinct strain.

The differences among the widely used laboratory strains AD169, Towne, and Toledo were localized to multiple ORFs in the UL/b′ region of the genome, encoding viral proteins with immunomodulatory or evasive functions [145,146]. Those genes that were lost upon extensive passaging in vitro played a crucial role in promoting viral replication and immune manipulation in vivo [147]. Consistently, extensive culture passaging led to the selection of HCMV mutants lacking these genes within weeks of propagation, and it also gave rise to variations between commonly used laboratory strains [146,148–150].

In the following years, with the development of more sensitive sequencing techniques, such as Sanger and high-throughput sequencing [151,152], a higher number of HCMV genomes were sequenced from bacterial artificial chromosomes [153–155], virion DNA [156], or overlapping PCR amplicons [148]. The widespread implementation of these new techniques allowed assessing different aspects of HCMV genome variation in clinical HCMV isolates from different cohorts of infected patients, thus providing novel insights into the genetic variation upon natural infection. Up to date, the complete genomes of 351 full-length HCMV strains have been published and analyzed (National Institute of Allergy and Infectious Diseases (NIAID)-sponsored Virus Pathogen Database and Analysis Resource (ViPR) [157]) [158]. Interestingly, these sequencing data show that HCMV can be highly polymorphic among and within hosts [159–161], with a high level of intra-host variability comparable to that of RNA viruses [159]. Given the fact that HCMV is a large double-stranded DNA virus, a high degree of genetic variation contradicted the logical expectation that the virus would retain high genome stability [162]. This unexpected intra-host HCMV diversity was initially attributed to the rapid occurrence of de novo mutations [159,160]—i.e. new mutations occur every time the virus infects a new host, thereby giving rise to a unique viral strain for each infected individual. Eventually, HCMV infection triggers a selection event where a new genotype becomes dominant due to the selective pressure of the immune response [159]. An alternative explanation was based on the evidence that viral and host factors can contribute to the onset of HCMV genome mutations, thus fostering virus genetic drift upon infection [163,164]. However, more recent data indicate that in non-mixed infections the mutation rate of HCMV is no different from that of other DNA viruses, while HCMV acquires a high degree of variability upon mixed infections [165–167], extensive recombination [152,166–168], or reactivation of the latent virus within a single individual. Many of these genetic alterations may in turn influence HCMV cell tropism, immune evasion, and disease outcomes. Indeed, the contribution of superinfection and recombination to viral genetic variability, an intensively debated topic, could have important ramifications in viral evolution, immune adaptation, and pathogenesis, especially in congenital or transplant patients [169,170].

Substantial efforts have been undertaken by various groups to correlate infection outcomes with variation in HCMV-specific genes [145,171,172]. Even though the selection of these genes was based on data supporting their potential role in viral pathogenicity and dissemination, these studies were only limited to Sanger sequencing of polymerase chain reaction (PCR) amplicons and often focused on a small number of polymorphic (hypervariable) genes. Furthermore, in such cases, low-abundance viral populations might have been missed, and the overall viral diversity underestimated. Thus, future studies should take advantage of high-throughput sequencing for fast detection and characterization of multiple-strain infections. Ideally, as recently put forward by Davison and co-workers, the definition of HCMV natural populations should be carried out by whole genome sequencing of HCMV strains directly from clinical samples [166].

6. Prevention

Although the development of an HCMV vaccine had already started in the 1970s, research in HCMV vaccine discovery received a major push when in 2000 the US Institute of Medicine placed HCMV among the top priorities for vaccine development [173]. Despite these increasing efforts, an effective vaccine against HCMV is currently missing, de facto leaving high-risk populations, chiefly immunocompromised patients and immunocompetent seronegative pregnant mothers, exposed to primary infection [174]. Given that one of the main obstacles to the development of an efficient vaccine against HCMV is the lack of protection against HCMV re-infection and/or reactivation, the first objective of a newly designed HCMV vaccine should be that of shielding vulnerable populations from primary infection. The long-range goal would then be to grant permanent protection against new infections with other HCMV strains and reactivated infections, which can occur repeatedly throughout life. To reach these goals, the ideal HCMV vaccine should be able to trigger a strong humoral response, in the form of binding and neutralizing antibodies, and an HCMV-specific $CD8^+$ and $CD4^+$ T-cell response. With this in mind, the experimental and clinical results achieved so far predict that the ideal HCMV vaccine should include: (i) gB, promoting both humoral—primarily antibody-binding—and T-cell-mediated response [164]; (ii) pp65, triggering a potent T-cell response; and (iii) the pentameric complex (PC), which prompts a quite strong neutralizing antibody (NAb) response [175]. Indeed, PC-induced NAbs are powerful cell-to-cell spread viral inhibitors in numerous cell types, not just fibroblasts. In the following sections, we summarize the most relevant approaches for designing effective HCMV vaccines (Table 2).

Table 2. HCMV Vaccines. NAb, Neutralizing Antibody; HELF, human embryonic lung fibroblasts; PC, pentameric complex; VLPs, virus like particles.

Live HCMV Vaccine	Description	Clinical Trials
Towne vaccine	HCMV attenuated strain.	Phase I/II clinical studies evidences: (a) no virus excretion; (b) no virus latency; (c) NAb induction; (d) generation of both HCM-specific CD4 and CD8 T-cell; (e) partial protection against a secondary infection.
Towne-Toledo chimera vaccines	Genetic recombinant Towne and Toledo.	In Phase I clinical trials they were well tolerated and with no virus excretion. One chimera was more immunogenic than Towne.
AD169 vaccine	HCMV attenuated strain.	Patients did not to display cell-mediated immunity depression or any systemic reactions.

Table 2. *Cont.*

Live HCMV Vaccine	Description	Clinical Trials
V160 vaccine	Replication-defective virus vaccine based on strain AD169.	Phase I study showed that V160-immunized HCMV-seronegative patients have features comparable in quality to those from seropositive subjects.
Viral vectored vaccines	Heterologous viral vectors used to deliver HCMV-encoded antigens: (a) canarypox virus vector; (b) alphavirus vector [Venezuelan equine encephalitis (VEE) virus]; (c) lymphocyte choriomeningitis virus vector; (d) modified vaccinia Ankara virus (MVA) vector; (e) adenovirus type 6 vector.	Many of them were tested in Phase I/II trials. While viral vectors cannot replicate completely when injected into humans, they have an optimal safety profile.
Non-living HCMV Vaccines		
gB subunit vaccines	Combination of the recombinant glycoprotein gB with an adjuvant.	Many Phase II studies showed that gB/MF59 vaccine had a certain degree of protection against HCMV infection through the mucosal route, but the antibody response was short-lived and disappeared within a year.
DNA based vaccines	Single or mixed combination of plasmids encoding viral antigens such as pp65, gB and IE1.	The most promising DNA-based plasmid vaccine, called ASP0113 divalent DNA vaccine, is currently in Phase III clinical trials.
RNA based vaccines	Different strategies: (a) synthetic self-amplifying mRNA expressing a pp65-IE1 construct and gB; (b) mRNA-based multiantigenic vaccine including pp65, gB and PC; (c) lipid nanoparticle (LNP)-encapsulated nucleoside-modified mRNA encoding full-length gB.	Currently they have been all tested only on animal models.
VLPs vaccines	Enveloped virus-like particles (VLPs) which exhibit on their surface gB and, in some cases, PC.	Different variations of VLPs have shown some success in animal immunization tests.
Dense body vaccines	Dense bodies purified from HCMV infected cell.	DB-injected mice did display both T-cell and NAb responses

6.1. Live HCMV Vaccines

The first attempts to develop an HCMV vaccine were focused on two attenuated strains: the AD169 and the Towne strain, both developed at the beginning of the 1970s by Elek and Stern in London and Plotkin in Philadelphia [176,177]. Their results show the vaccine to be very safe due to the absence of virus excretions—this was true even for vaccinated kidney transplant patients on chronic immunosuppression. Furthermore, these patients did not display cell-mediated immunity depression or any systemic reactions. Concerning immunogenicity, NAbs were present at levels similar to those found in the serum of patients infected with the wild type virus. Although the vaccine was able to generate both HCMV-specific $CD8^+$ and $CD4^+$ T-cell response, the immunity induced by vaccination

did not prevent secondary infection in pregnant women, while mucosal immunity induced by natural infection did. Thus, immunization was deemed incomplete [178]. A reason for this only partial success is probably related to the fact that the two HCMV strains used to obtain the vaccines, when propagated on fibroblasts in vitro, are known to acquire mutations in *UL128L*, a subunit of the pentameric complex. In particular, the Towne strain undergoes a 2-bp insertion (TT), causing a frameshift mutation in *UL130* [154]. This event is particularly important if we consider that a functional PC is required for the virus entry into epithelial cells or ECs, whereas gB and the gH/gL dimer are sufficient for entry into fibroblasts [179]. Consistently, epithelial-neutralizing antibodies against Towne strain, harboring a mutation in PC, were 28-fold lower than those induced by natural infection [180].

To develop a live HCMV vaccine with the same safety of the Towne's one but more protective and immunogenic, four genetic recombinants of Towne and Toledo, a low-passage HCMV strain, were subsequently generated. The chimera vaccine candidates were tested in Phase I clinical trials and found to be well tolerated and with no virus excretions in saliva or urine, with two chimeras (2 and 4) being more immunogenic than Towne [181].

In 2012, a replication-defective virus strategy was put in place for the first time. It consisted of restoring a mutated PC through serial passages of AD169-infected ECs. Subsequently, the two HCMV proteins IE1/2 and UL51 were linked to a protein domain that rendered their stability dependent on the synthetic compound Shield 1 (Shld1). That meant that HCMV could replicate normally in the presence of Shld1, whereas in the absence of such molecule viral replication was abortive due to the IE1/2 and UL51 degradation. As later confirmed in several studies, this defective virus, named V160, retained its ability to express all other AD169-specific proteins, including PC and gB, thereby eliciting both T cell and humoral responses in non-human primates as well as in humans [182]. A recently concluded Phase I study showed that sera from V160-immunized HCMV-seronegative patients have features similar in quality to those from seropositive subjects, justifying future clinical trials on this vaccine [183].

Finally, several viral vectored vaccines against HCMV have been formulated and tested in Phase I/II trials. These strategies for vaccine development employ heterologous viral vectors to deliver HCMV-encoded antigens such as gB, pp65, IE1, and, in some cases, PC proteins. While viral vectors cannot replicate completely when injected into humans, they have an optimal safety profile and can efficiently carry the desired viral antigens. The following heterologous viral vectors were tested as HCMV vaccines: canarypox virus vector, alphavirus vector (Venezuelan equine encephalitis (VEE) virus), lymphocyte choriomeningitis virus vector, modified vaccinia Ankara virus (MVA) vector, and adenovirus type 6 vector [184].

In conclusion, although consistent progress has been made over the past 45 years, and several potential candidates have emerged, an effective live HCMV vaccine has yet to be successfully developed.

6.2. Non-Living HCMV Vaccines

The simplest non-living HCMV vaccines are the so-called subunit vaccines, obtained by combining subunit immunogens with an adjuvant. The first one was made with the recombinant glycoprotein gB with the microfluidized adjuvant 59 (MF59) [185]. Several Phase II clinical trials in both adults and toddlers showed that after three doses given at 0, 1, and 6 months gB/MF59 was able to induce a reasonable NAb titer. Even though these studies showed that gB when injected alone as a vaccine had a certain degree of protection against HCMV infection through the mucosal route, the antibody response was short-lived and disappeared within a year [186].

A different strategy for non-living vaccines is based on genetic programming of host cells—via uptake of naked DNA or RNA—to express the desired immunogens in vivo. In this regard, a plasmid-based DNA vaccine, called the ASP0113 bivalent DNA vaccine, managed to reach Phase III clinical trials. This vaccine was obtained using two plasmids: VCL-6365, encoding the extracellular domain of gB, and VCL-6368, encoding a modified pp65 protein kinase gene [187].

Likewise, the use of self-replicating RNA to express viral proteins has made progress in recent years. Specifically, an mRNA-based multiantigenic vaccine containing gB, pp65, and PC injected in mice was capable of inducing potent cell-mediated and humoral immune responses. Although an effective cell response to pp65 was hampered by the presence of other HCMV antigens, it could be restored by sequential immunization of pp65 followed by PC + gB + pp65 [188]. In a very recent study a new RNA-based vaccine, consisting of lipid nanoparticle (LNP)-encapsulated nucleoside-modified mRNA encoding full-length gB, was tested on a group of young New Zealand White rabbits. The gB nucleoside-modified mRNA-LNP vaccines were highly immunogenic with similar kinetics and comparable peak gB-binding and functional antibody responses induced by gB/MF59 subunit vaccine. However, rabbits immunized with nucleoside-modified mRNA-LNP showed significantly longer duration of vaccine-induced antibody responses [189].

Other heavily studied approaches include the use of HCMV peptides, to elicit cellular immunity in transplant patients, and enveloped virus-like particles (VLPs) in order to stimulate wild type viruses in the absence of viral DNA, with VLPs exhibiting on their surface gB and, in some cases, PC different variations of VLPs have had some success in animal immunization tests [190,191].

One different approach involves the purification of dense bodies (DBs), including virion tegument and envelope proteins but not viral genome, produced by HCMV-infected cells. DB-injected mice did display both T-cell and NAb responses [192].

In summary, the antigens needed for successful vaccination against HCMV are well known from the literature. Thus, further effort will be required in combining such antigens to achieve a durable response that would protect an individual for an extended time.

7. Treatment

The high incidence and clinical manifestations of HCMV infection underscore the need for efficient antiviral therapies in treating disease in immunocompromised patients and congenitally infected infants.

Four compounds are currently approved for systemic treatment or prophylaxis of HCMV infections: ganciclovir (GCV) and its oral prodrug valganciclovir (VGCV), cidofovir (CDV), foscarnet (FOS), and, more recently, letermovir (LTV) [193,194] (Table 3).

Table 3. Antiviral agents approved for treatment or prevention of HCMV infections.

Agent	Compound Information	Viral Target	Mechanism of Action	Route of Administration	Dosage	Toxicities
Ganciclovir (Cytovene®)	Acyclic nucleoside analogue of guanine	UL54	When phosphorylated to Ganciclovir triphosphate inhibits the viral DNA polymerase UL54	Intravenous	Induction: 5 mg/kg every 12 hours for 7–14 days Maintenance: 5 mg/kg for 100–120 days after transplant	Neutropenia Thrombocytopenia Neurotoxicity
				Oral	FDA approved for maintenance therapy only using 1 mg three times a day	
Valganciclovir (Valcyte®)	L-Valyl ester of Ganciclovir	UL54	Converted to Ganciclovir in intestine and liver, inhibits the viral DNA polymerase	Oral	Induction: 900 mg twice a day for 21 days Maintenance: 900 mg once per day	Granulocytopenia Anemia Thrombocytopenia
Cidofovir (Vistide®)	Deoxycytidine acyclic nucleotide phosphonate analog	UL54	When phosphorylated to Cidofovir biphosphate inhibits the viral DNA polymerase	Intravenous	Induction: 5 mg/kg weekly for 2 weeks Maintenance: 5 mg/kg every 2 weeks	Nephrotoxicity Metabolic acidosis Ocular hypotony

Table 3. *Cont.*

Agent	Compound Information	Viral Target	Mechanism of Action	Route of Administration	Dosage	Toxicities
Foscarnet (Foscavir®)	Synthetic organic analogue of inorganic pyrophosphate	UL54	Inhibits activity of pyrophosphate binding site on viral DNA polymerase UL54	Intravenous	Induction: 60 mg/kg every 8 hours for 14–21 days Maintenance: 90–120 mg/kg everyday	Nephrotoxicity Hypocalcemia Electrolytes imbalance Genital ulceration
Letermovir (Prevymis®)	Non-nucleoside, 3,4-dihydroquinazolinyl acetic	pUL56	Binds pUL56 subunit of the HCMV terminase complex preventing the cleavage of concatemeric DNA	Intravenous Oral	Prophylaxis: 480 mg once a day, through 100 days post-transplant	

Among the latter, GCV, CDV, and FOS exhibit similar activities, as they target viral DNA polymerases, consequently inhibiting the synthesis of HCMV DNA [195]. In contrast, LTV blocks DNA packaging in the viral capsid by interfering with the viral terminase complex [196]. Furthermore, in the late 1990s, a fifth antiviral compound, fomivirsen, also known as Vitravene, was approved for the treatment of HCMV infection by the Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medicinal Products (EMEA). Fomivirsen, an antisense 21-mer phosphorothioate oligonucleotide (5′-GCGTTTGCTCTTCTTCTTGCG-3′) complementary to the mRNA encoding the IE-2 protein, is used to treat HCMV-induced retinopathy in immunocompromised subjects [197]. Prophylactic treatment with acyclovir has also been approved in some countries, although the effectiveness of this approach is moderate.

The front-line therapy for HCMV infections is GCV, which was approved for medical use in 1988 and is now routinely used to treat congenitally HCMV-infected infants [198] and immunocompromised hosts with HCMV disease [199] or given as a prophylactic agent to prevent HCMV disease [194,200,201]. GCV, a monosodium salt of 9-(1,3-dihydroxy-2-propoxymethyl)-guanine, is an acyclic nucleoside analog administered by intravenous infusion that, when phosphorylated to form GCV triphosphate (GCV-TP), inhibits the viral UL54 DNA polymerase and, by competing with deoxyguanosine (dGTP), curbs the elongation of viral DNA. GCV is administered in an inactive form and is selectively phosphorylated in the infected cells by the HCMV UL97 kinase [202]. This kinase is responsible for the conversion of GCV to GCV monophosphate (GCV-MP), while the subsequent phosphorylation steps generating the active form of the drug (GCV-TP) are controlled by cellular kinases [203]. However, its moderate antiviral activity and dose-limiting toxicity hinder its efficacy and may lead to the development of drug-resistant infections, mainly in immunocompromised patients [204]. Specifically, GCV-induced cytotoxicity may result in thrombocytopenia and neutropenia in transplant recipients [205].

Secondary therapies for HCMV infections consist of CDV and FOS, which can both cause nephrotoxicity and give rise to resistant infections [193,194].

CDV was approved in 1996 for the treatment of HCMV retinitis in AIDS patients and is available only intravenously. Cidofovir, 1-[(S)-3-hydroxy-2-(phosphonomethoxy) propyl]cytosine, is an acyclic phosphonate nucleoside (ANP) acting as an analog of CMP (cytidine monophosphate). CDV presents a broad spectrum of antiviral activity; in particular, it acts against most DNA viruses (e.g., herpesviruses, adenovirus, polyomavirus, and orthopoxvirus) [206]. Since CDV is a monophosphate analog, it does not require the initial activating phosphorylation by the UL97 viral kinase. Nevertheless, to be active, CDV needs to be diphosphorylated by the pyruvate kinase, creatine kinase and nucleoside diphosphate kinase, all present at high levels in infected HCMV cells. Once activated, CDV competes with deoxycytidine triphosphate and is incorporated in the DNA as an alternative substrate of the viral DNA polymerase where it acts as a non-mandatory chain terminator [206]. CDV resistance is

only associated with viral DNA polymerase mutations and occurs at a frequency comparable to that of GCV [206].

FOS was approved for the treatment of HCMV infections in 1991. Similar to CDV, FOS inhibits the replication of several types of DNA viruses (e.g., several herpes family viruses, hepatitis B virus, and HIV), but it is primarily used for the treatment of HCMV retinitis [207]. FOS is a synthetic organic analog of inorganic pyrophosphate, which reversibly inhibits the activity of the UL54 DNA polymerase. FOS resistance during long-term therapy occurs at similar rates to those displayed by CDV and GCV. However, FOS can be particularly useful against some GCV-resistant infections as the frequency of GCV cross-resistance is much lower than that observed for CDV and GCV, even though renal toxicity may limit its usefulness [208]. Combination therapy with GCV has also been investigated, but it does not seem to be more efficient than GCV alone. Despite the above limitations, the broad spectrum of antiviral activity of FOS makes this compound useful for the treatment of some GCV-resistant infections. Of note, FOS has also been approved for the treatment of ACV-resistant HSV infections. Lastly, because this agent crosses the blood-brain barrier, it is indicated for the treatment of viral infections involving the central nervous system.

Drug resistance results from the development of single or multiple mutations leading to different levels of resistance that can reduce the efficacy of the antiviral treatments. Since all these drugs, except of fomivirsen, target, directly or indirectly, viral DNA polymerase, the emergence of drug-resistant HCMV strains, often due to mutations in UL97 and/or UL54, has increasingly hampered disease management [209]. Therefore, there is an urgent medical need for new anti-HCMV agents with new mechanisms of action and fewer side effects.

Currently, a promising new class of riboside analogs with strong and specific antiviral activity against HCMV is being tested in a clinical trial. One of these drugs is maribavir, a benzimidazole riboside characterized by a novel mechanism of action based on its ability to inhibit UL97, a viral enzyme that blocks nuclear egress of viral capsids, and to interfere with viral DNA synthesis. Even though clinical trials with maribavir have not yet been conclusive, the drug is still being evaluated as a potential preventive treatment of HCMV infection [210,211].

BAY 38-4766, also called tomeglovir, is a non-nucleoside antiviral. The antiviral activity of BAY 38-4766 is due to its ability to hinder DNA maturation most likely by targeting the UL89 and UL56 genes encoding for the subunits of a multiprotein complex involved in HCMV termination [212]. Interestingly, in a guinea pig model, after infection and treatment, measurable amounts of the compound were detected in fetal blood, attesting that the drug is capable of crossing the placenta in pregnant animals [213]. BAY 38-4766 is in clinical development and has shown a positive safety profile in healthy male volunteers at single oral doses of up to 2000 mg. However, no recent studies have highlighted the current state of clinical development of this drug or related compounds in the series [213].

GW275175X is a new benzimidazole riboside class of HCMV inhibitors that can block the maturational cleavage of high molecular weight HCMV DNA by interacting with pUL56 and pUL89, the two subunits of the HCMV terminase complex [214]. GW275175X was advanced to Phase I clinical trial with an increasing dose of safety, pharmacokinetics, and tolerability, but was later set aside to prioritize maribavir testing. The clinical potential of this antiviral still requires further study.

Brincidofovir (CMX001), a prodrug of CDV, is an oral lipid-drug conjugate quickly absorbed into cells whereupon the lipid side chain is cleaved, releasing CDV to be further phosphorylated by intracellular kinases to CDV diphosphate. Since brincidofovir is not a substrate for the oxyanion transporter in the kidney, kidney damage should be reduced. Nonetheless, CDV may lead to gastrointestinal toxicity, which is quite often dose-limiting [215].

In 2017, LTV became the latest FDA-approved drug for prophylaxis of HCMV infections in allogeneic HSCT recipients [216]. LTV is a non-nucleoside 3,4-dihydroquinazolinyl acetic acid, which can be administered orally or intravenously infused. LTV acts as an inhibitor of the HCMV DNA terminase complex by interacting with the pUL56 subunit of such complex and preventing the cleavage of concatemeric DNA into monomeric genome length DNA, which ultimately inhibits DNA

packaging into the virion. LVT displays good efficacy against different clinical isolates of HCMV, including GCV-resistant strains, and is ineffective against all other herpesviruses [217].

To sum up, evidence from the literature indicates that currently available anti-HCMV drugs, despite being able to interfere with viral replication through different mechanisms of action, are often associated with multiple side effects such as drug toxicity, poor oral bioavailability, and drug resistance. Therefore, innovative compounds targeting new virus components with fewer adverse effects are urgently needed to improve patient outcomes.

8. Conclusions

HCMV is the leading cause of congenital infections resulting in severe morbidity and mortality among newborns worldwide. This virus is highly polymorphic, particularly in genes contributing to immune modulation. Despite a large amount of HCMV research over the past few decades, the mechanisms and virulence factors contributing to HCMV pathogenesis and particular clinical outcomes remain unclear. To make matters worse, to date, no safe vaccines against HCMV infections exist and current antiviral therapies are quite unsatisfactory due to the frequent occurrence of drug resistance and toxicity.

Overall, the lack of advances in the treatment of HCMV-driven diseases clearly clashes with the widespread notion that HCMV poses "a greater threat to infants than other viruses". Thus, an in-depth understanding of HCMV-host interactions, especially at the individual level, will be instrumental to develop new diagnostic/therapeutic tools for the clinical management of this viral disease.

Author Contributions: Conceptualization, M.B.; writing—original draft preparation, M.B. and F.G; writing—review and editing, M.B., F.G., A.C., G.G. (Gloria Griffante), G.G. (Ganna Galitska), S.P., and C.A.; supervision, M.B.; and funding acquisition, M.B., F.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Research Funding from the University of Turin, 2019 (RILO1901), to M.B. and F.G.

Acknowledgments: We thank Marcello Arsura for critically reviewing the manuscript.

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

References

1. Arvin, A.; Campadelli-Fiume, G.; Mocarski, E.; Moore, P.S.; Roizman, B.; Whitley, R.; Yamanishi, K. *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis*; Cambridge University Press: Cambridge, UK, 2007; ISBN 978-0-521-82714-0.
2. Sinzger, C.; Digel, M.; Jahn, G. Cytomegalovirus cell tropism. *Curr. Top. Microbiol. Immunol.* **2008**, *325*, 63–83. [CrossRef]
3. Griffiths, P.; Baraniak, I.; Reeves, M. The pathogenesis of human cytomegalovirus. *J. Pathol.* **2015**, *235*, 288–297. [CrossRef] [PubMed]
4. Landolfo, S.; Gariglio, M.; Gribaudo, G.; Lembo, D. The human cytomegalovirus. *Pharmacol. Ther.* **2003**, *98*, 269–297. [CrossRef]
5. Zuhair, M.; Smit, G.S.A.; Wallis, G.; Jabbar, F.; Smith, C.; Devleesschauwer, B.; Griffiths, P. Estimation of the worldwide seroprevalence of cytomegalovirus: A systematic review and meta-analysis. *Rev. Med. Virol.* **2019**, *29*, e2034. [CrossRef] [PubMed]
6. Crough, T.; Khanna, R. Immunobiology of human cytomegalovirus: From bench to bedside. *Clin. Microbiol. Rev.* **2009**, *22*, 76–98. [CrossRef]
7. Mestas, E. Congenital Cytomegalovirus. *Adv. Neonat. Care* **2016**, *16*, 60–65. [CrossRef]
8. Mocarski, E.S.; Shenk, T.; Pass, R. Cytomegaloviruses. In *Fields Virology*; Knipe, D.M., Howley, P.M., Eds.; Lippincott Williams & Wilkins: Philadelphia, PA, USA, 2007; pp. 2701–2772.
9. Bate, S.L.; Dollard, S.C.; Cannon, M.J. Cytomegalovirus seroprevalence in the United States: The national health and nutrition examination surveys, 1988–2004. *Clin. Infect. Dis.* **2010**, *50*, 1439–1447. [CrossRef]

10. Horwitz, C.A.; Henle, W.; Henle, G.; Snover, D.; Rudnick, H.; Balfour, H.H.; Mazur, M.H.; Watson, R.; Schwartz, B.; Muller, N. Clinical and laboratory evaluation of cytomegalovirus-induced mononucleosis in previously healthy individuals. Report of 82 cases. *Medicine* **1986**, *65*, 124–134. [CrossRef]
11. Cohen, J.I.; Corey, G.R. Cytomegalovirus infection in the normal host. *Medicine* **1985**, *64*, 100–114. [CrossRef]
12. Nangle, S.; Mitra, S.; Roskos, S.; Havlichek, D. Cytomegalovirus infection in immunocompetent adults: Is observation still the best strategy? *IDCases* **2018**, *14*, e00442. [CrossRef]
13. Urbano, J.; Moreira-Silva, S.; Frioes, F.; Almeida, J.; Abreu, C.; Pimenta, J. Cytomegalic Infection in an Immunocompetent Patient: A Case With Multiple Organ Dysfunction. *J. Med. Cases* **2015**, *6*, 480–482. [CrossRef]
14. Fakhreddine, A.Y.; Frenette, C.T.; Konijeti, G.G. A Practical Review of Cytomegalovirus in Gastroenterology and Hepatology. *Gastroenterol. Res. Pract.* **2019**, *2019*, 6156581. [CrossRef] [PubMed]
15. Rafailidis, P.I.; Mourtzoukou, E.G.; Varbobitis, I.C.; Falagas, M.E. Severe cytomegalovirus infection in apparently immunocompetent patients: A systematic review. *Virol. J.* **2008**, *5*, 47. [CrossRef] [PubMed]
16. Dioverti, M.V.; Razonable, R.R. Cytomegalovirus. *Microbiol. Spectr.* **2016**, *4*. [CrossRef] [PubMed]
17. Gianella, S.; Massanella, M.; Wertheim, J.O.; Smith, D.M. The Sordid Affair Between Human Herpesvirus and HIV. *J. Infect. Dis.* **2015**, *212*, 845–852. [CrossRef] [PubMed]
18. Steininger, C.; Puchhammer-Stöckl, E.; Popow-Kraupp, T. Cytomegalovirus disease in the era of highly active antiretroviral therapy (HAART). *J. Clin. Virol.* **2006**, *37*, 1–9. [CrossRef] [PubMed]
19. Freeman, M.L.; Mudd, J.C.; Shive, C.L.; Younes, S.-A.; Panigrahi, S.; Sieg, S.F.; Lee, S.A.; Hunt, P.W.; Calabrese, L.H.; Gianella, S.; et al. CD8 T-Cell Expansion and Inflammation Linked to CMV Coinfection in ART-treated HIV Infection. *Clin. Infect. Dis.* **2016**, *62*, 392–396. [CrossRef]
20. Hunt, P.W.; Martin, J.N.; Sinclair, E.; Epling, L.; Teague, J.; Jacobson, M.A.; Tracy, R.P.; Corey, L.; Deeks, S.G. Valganciclovir reduces T cell activation in HIV-infected individuals with incomplete CD4+ T cell recovery on antiretroviral therapy. *J. Infect. Dis.* **2011**, *203*, 1474–1483. [CrossRef]
21. Chiotan, C.; Radu, L.; Serban, R.; Cornăcel, C.; Cioboata, M.; Anghel, A. Cytomegalovirus retinitis in HIV/AIDS patients. *J. Med. Life* **2014**, *7*, 237–240.
22. Ho, M.; Invernizzi, A.; Zagora, S.; Tsui, J.; Oldani, M.; Lui, G.; McCluskey, P.; Young, A.L. Presenting Features, Treatment and Clinical Outcomes of Cytomegalovirus Retinitis: Non-HIV Patients Vs HIV Patients. *Ocul. Immunol. Inflamm.* **2019**, 1–8. [CrossRef]
23. Jabs, D.A. Cytomegalovirus retinitis and the acquired immunodeficiency syndrome—Bench to bedside: LXVII Edward Jackson Memorial Lecture. *Am. J. Ophthalmol.* **2011**, *151*, 198–216.e1. [CrossRef] [PubMed]
24. Meiselman, M.S.; Cello, J.P.; Margaretten, W. Cytomegalovirus colitis. Report of the clinical, endoscopic, and pathologic findings in two patients with the acquired immune deficiency syndrome. *Gastroenterology* **1985**, *88*, 171–175. [CrossRef]
25. Springer, K.L.; Weinberg, A. Cytomegalovirus infection in the era of HAART: Fewer reactivations and more immunity. *J. Antimicrob. Chemother.* **2004**, *54*, 582–586. [CrossRef] [PubMed]
26. Humar, A.; Snydman, D. AST Infectious Diseases Community of Practice Cytomegalovirus in solid organ transplant recipients. *Am. J. Transplant.* **2009**, *9*, S78–S86. [CrossRef] [PubMed]
27. Azevedo, L.S.; Pierrotti, L.C.; Abdala, E.; Costa, S.F.; Strabelli, T.M.V.; Campos, S.V.; Ramos, J.F.; Latif, A.Z.A.; Litvinov, N.; Maluf, N.Z.; et al. Cytomegalovirus infection in transplant recipients. *Clinics* **2015**, *70*, 515–523. [CrossRef]
28. Simon, D.M.; Levin, S. Infectious complications of solid organ transplantations. *Infect. Dis. Clin. North Am.* **2001**, *15*, 521–549. [CrossRef]
29. Razonable, R.R.; Humar, A. Cytomegalovirus in solid organ transplant recipients-Guidelines of the American Society of Transplantation Infectious Diseases Community of Practice. *Clin. Transplant.* **2019**, *33*, e13512. [CrossRef]
30. Cho, S.-Y.; Lee, D.-G.; Kim, H.-J. Cytomegalovirus Infections after Hematopoietic Stem Cell Transplantation: Current Status and Future Immunotherapy. *Int. J. Mol. Sci.* **2019**, *20*, 2666. [CrossRef]
31. Travi, G.; Pergam, S.A. Cytomegalovirus pneumonia in hematopoietic stem cell recipients. *J. Intensiv. Care Med.* **2014**, *29*, 200–212. [CrossRef]
32. Lönnqvist, B.; Ringdèn, O.; Ljungman, P.; Wahren, B.; Gahrton, G. Reduced risk of recurrent leukaemia in bone marrow transplant recipients after cytomegalovirus infection. *Br. J. Haematol.* **1986**, *63*, 671–679. [CrossRef]

33. Kollman, C.; Howe, C.W.; Anasetti, C.; Antin, J.H.; Davies, S.M.; Filipovich, A.H.; Hegland, J.; Kamani, N.; Kernan, N.A.; King, R.; et al. Donor characteristics as risk factors in recipients after transplantation of bone marrow from unrelated donors: The effect of donor age. *Blood* **2001**, *98*, 2043–2051. [CrossRef] [PubMed]
34. Ljungman, P.; Brand, R.; Einsele, H.; Frassoni, F.; Niederwieser, D.; Cordonnier, C. Donor CMV serologic status and outcome of CMV-seropositive recipients after unrelated donor stem cell transplantation: An EBMT megafile analysis. *Blood* **2003**, *102*, 4255–4260. [CrossRef] [PubMed]
35. Behrendt, C.E.; Rosenthal, J.; Bolotin, E.; Nakamura, R.; Zaia, J.; Forman, S.J. Donor and recipient CMV serostatus and outcome of pediatric allogeneic HSCT for acute leukemia in the era of CMV-preemptive therapy. *Biol. Blood Marrow Transplant.* **2009**, *15*, 54–60. [CrossRef] [PubMed]
36. Farina, L.; Bruno, B.; Patriarca, F.; Spina, F.; Sorasio, R.; Morelli, M.; Fanin, R.; Boccadoro, M.; Corradini, P. The hematopoietic cell transplantation comorbidity index (HCT-CI) predicts clinical outcomes in lymphoma and myeloma patients after reduced-intensity or non-myeloablative allogeneic stem cell transplantation. *Leukemia* **2009**, *23*, 1131–1138. [CrossRef] [PubMed]
37. Schmidt-Hieber, M.; Labopin, M.; Beelen, D.; Volin, L.; Ehninger, G.; Finke, J.; Socié, G.; Schwerdtfeger, R.; Kröger, N.; Ganser, A.; et al. CMV serostatus still has an important prognostic impact in de novo acute leukemia patients after allogeneic stem cell transplantation: A report from the Acute Leukemia Working Party of EBMT. *Blood* **2013**, *122*, 3359–3364. [CrossRef]
38. Elmaagacli, A.H.; Steckel, N.K.; Koldehoff, M.; Hegerfeldt, Y.; Trenschel, R.; Ditschkowski, M.; Christoph, S.; Gromke, T.; Kordelas, L.; Ottinger, H.D.; et al. Early human cytomegalovirus replication after transplantation is associated with a decreased relapse risk: Evidence for a putative virus-versus-leukemia effect in acute myeloid leukemia patients. *Blood* **2011**, *118*, 1402–1412. [CrossRef]
39. Jang, J.E.; Kim, S.J.; Cheong, J.-W.; Hyun, S.Y.; Kim, Y.D.; Kim, Y.R.; Kim, J.S.; Min, Y.H. Early CMV replication and subsequent chronic GVHD have a significant anti-leukemic effect after allogeneic HSCT in acute myeloid leukemia. *Ann. Hematol.* **2015**, *94*, 275–282. [CrossRef]
40. Green, M.L.; Leisenring, W.M.; Xie, H.; Walter, R.B.; Mielcarek, M.; Sandmaier, B.M.; Riddell, S.R.; Boeckh, M. CMV reactivation after allogeneic HCT and relapse risk: Evidence for early protection in acute myeloid leukemia. *Blood* **2013**, *122*, 1316–1324. [CrossRef]
41. Mariotti, J.; Maura, F.; Spina, F.; Roncari, L.; Dodero, A.; Farina, L.; Montefusco, V.; Carniti, C.; Sarina, B.; Patriarca, F.; et al. Impact of cytomegalovirus replication and cytomegalovirus serostatus on the outcome of patients with B cell lymphoma after allogeneic stem cell transplantation. *Biol. Blood Marrow Transplant.* **2014**, *20*, 885–890. [CrossRef]
42. Yanada, M.; Yamamoto, K.; Emi, N.; Naoe, T.; Suzuki, R.; Taji, H.; Iida, H.; Shimokawa, T.; Kohno, A.; Mizuta, S.; et al. Cytomegalovirus antigenemia and outcome of patients treated with pre-emptive ganciclovir: Retrospective analysis of 241 consecutive patients undergoing allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant.* **2003**, *32*, 801–807. [CrossRef]
43. Jeljeli, M.; Guérin-El Khourouj, V.; Porcher, R.; Fahd, M.; Leveillé, S.; Yakouben, K.; Ouachée-Chardin, M.; LeGoff, J.; Cordeiro, D.J.; Pédron, B.; et al. Relationship between cytomegalovirus (CMV) reactivation, CMV-driven immunity, overall immune recovery and graft-versus-leukaemia effect in children. *Br. J. Haematol.* **2014**, *166*, 229–239. [CrossRef] [PubMed]
44. Peric, Z.; Wilson, J.; Durakovic, N.; Ostojic, A.; Desnica, L.; Vranjes, V.R.; Marekovic, I.; Serventi-Seiwerth, R.; Vrhovac, R. Early human cytomegalovirus reactivation is associated with lower incidence of relapse of myeloproliferative disorders after allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant.* **2018**, *53*, 1450–1456. [CrossRef] [PubMed]
45. Manjappa, S.; Bhamidipati, P.K.; Stokerl-Goldstein, K.E.; DiPersio, J.F.; Uy, G.L.; Westervelt, P.; Liu, J.; Schroeder, M.A.; Vij, R.; Abboud, C.N.; et al. Protective effect of cytomegalovirus reactivation on relapse after allogeneic hematopoietic cell transplantation in acute myeloid leukemia patients is influenced by conditioning regimen. *Biol. Blood Marrow Transplant.* **2014**, *20*, 46–52. [CrossRef] [PubMed]
46. Wang, C.; Zhang, X.; Bialek, S.; Cannon, M.J. Attribution of congenital cytomegalovirus infection to primary versus non-primary maternal infection. *Clin. Infect. Dis.* **2011**, *52*, e11–e13. [CrossRef] [PubMed]
47. Goderis, J.; De Leenheer, E.; Smets, K.; Van Hoecke, H.; Keymeulen, A.; Dhooge, I. Hearing loss and congenital CMV infection: A systematic review. *Pediatrics* **2014**, *134*, 972–982. [CrossRef] [PubMed]

48. Coppola, T.; Mangold, J.F.; Cantrell, S.; Permar, S.R. Impact of Maternal Immunity on Congenital Cytomegalovirus Birth Prevalence and Infant Outcomes: A Systematic Review. *Vaccines* **2019**, *7*, 129. [CrossRef]
49. PRISMA. Available online: http://www.prisma-statement.org/ (accessed on 23 April 2020).
50. Lanzieri, T.M.; Dollard, S.C.; Bialek, S.R.; Grosse, S.D. Systematic review of the birth prevalence of congenital cytomegalovirus infection in developing countries. *Int. J. Infect. Dis.* **2014**, *22*, 44–48. [CrossRef]
51. Fowler, K.B.; Boppana, S.B. Congenital cytomegalovirus infection. *Semin. Perinatol.* **2018**, *42*, 149–154. [CrossRef]
52. Stagno, S.; Pass, R.F.; Dworsky, M.E.; Britt, W.J.; Alford, C.A. Congenital and perinatal cytomegalovirus infections: Clinical characteristics and pathogenic factors. *Birth Defects Orig. Artic. Ser.* **1984**, *20*, 65–85.
53. Schopfer, K.; Lauber, E.; Krech, U. Congenital cytomegalovirus infection in newborn infants of mothers infected before pregnancy. *Arch. Dis. Child.* **1978**, *53*, 536–539. [CrossRef]
54. Diosi, P.; Babusceac, L.; Nevinglovschi, O.; Kun-Stoicu, G. Cytomegalovirus infection associated with pregnancy. *Lancet* **1967**, *2*, 1063–1066. [CrossRef]
55. Dworsky, M.; Yow, M.; Stagno, S.; Pass, R.F.; Alford, C. Cytomegalovirus infection of breast milk and transmission in infancy. *Pediatrics* **1983**, *72*, 295–299. [PubMed]
56. Hamprecht, K.; Maschmann, J.; Vochem, M.; Dietz, K.; Speer, C.P.; Jahn, G. Epidemiology of transmission of cytomegalovirus from mother to preterm infant by breastfeeding. *Lancet* **2001**, *357*, 513–518. [CrossRef]
57. Stagno, S.; Reynolds, D.W.; Pass, R.F.; Alford, C.A. Breast milk and the risk of cytomegalovirus infection. *N. Engl. J. Med.* **1980**, *302*, 1073–1076. [CrossRef] [PubMed]
58. Meyers, J.D. Cytomegalovirus infection following marrow transplantation: Risk, treatment, and prevention. *Birth Defects Orig. Artic. Ser.* **1984**, *20*, 101–117.
59. Van den Berg, A.P.; van Son, W.J.; Jiwa, N.M.; van der Bij, W.; Schirm, J.; van der Giessen, M.; The, T.H. Recent advances in the diagnosis of active cytomegalovirus infection after organ transplantation. *Transplant. Proc.* **1990**, *22*, 226–228. [PubMed]
60. Adler, S.P. Cytomegalovirus and child day care: Risk factors for maternal infection. *Pediatr. Infect. Dis. J.* **1991**, *10*, 590–594. [CrossRef]
61. Staras, S.A.S.; Dollard, S.C.; Radford, K.W.; Flanders, W.D.; Pass, R.F.; Cannon, M.J. Seroprevalence of cytomegalovirus infection in the United States, 1988–1994. *Clin. Infect. Dis.* **2006**, *43*, 1143–1151. [CrossRef]
62. Handsfield, H.H.; Chandler, S.H.; Caine, V.A.; Meyers, J.D.; Corey, L.; Medeiros, E.; McDougall, J.K. Cytomegalovirus infection in sex partners: Evidence for sexual transmission. *J. Infect. Dis.* **1985**, *151*, 344–348. [CrossRef]
63. Farrell, H.E.; Lawler, C.; Tan, C.S.E.; MacDonald, K.; Bruce, K.; Mach, M.; Davis-Poynter, N.; Stevenson, P.G. Murine Cytomegalovirus Exploits Olfaction To Enter New Hosts. *MBio* **2016**, *7*, e00251-16. [CrossRef]
64. Britt, W. Virus entry into host, establishment of infection, spread in host, mechanisms of tissue damage. In *Human Herpesviruses: Biology, Therapy and Immunoprophylaxis*; Arvin, A., Campadelli-Fiume, G., Mocarski, E., Moore, P.S., Roizman, B., Whitley, R., Yamanishi, K., Eds.; Cambridge University Press: Cambridge, UK, 2007; ISBN 978-0-521-82714-0.
65. Collins, T.M.; Quirk, M.R.; Jordan, M.C. Biphasic viremia and viral gene expression in leukocytes during acute cytomegalovirus infection of mice. *J. Virol.* **1994**, *68*, 6305–6311. [CrossRef] [PubMed]
66. Jackson, J.W.; Sparer, T. There Is Always Another Way! Cytomegalovirus' Multifaceted Dissemination Schemes. *Viruses* **2018**, *10*, 383. [CrossRef] [PubMed]
67. Boom, R.; Sol, C.J.A.; Schuurman, T.; Van Breda, A.; Weel, J.F.L.; Beld, M.; Ten Berge, I.J.M.; Wertheim-Van Dillen, P.M.E.; De Jong, M.D. Human cytomegalovirus DNA in plasma and serum specimens of renal transplant recipients is highly fragmented. *J. Clin. Microbiol.* **2002**, *40*, 4105–4113. [CrossRef] [PubMed]
68. Chen, X.Y.; Hou, P.F.; Bi, J.; Ying, C.M. Detection of human cytomegalovirus DNA in various blood components after liver transplantation. *Braz. J. Med. Biol. Res.* **2014**, *47*, 340–344. [CrossRef]
69. Eisenfeld, L.; Silver, H.; McLaughlin, J.; Klevjer-Anderson, P.; Mayo, D.; Anderson, J.; Herson, V.; Krause, P.; Savidakis, J.; Lazar, A. Prevention of transfusion-associated cytomegalovirus infection in neonatal patients by the removal of white cells from blood. *Transfusion* **1992**, *32*, 205–209. [CrossRef]
70. Heldwein, E.E. gH/gL supercomplexes at early stages of herpesvirus entry. *Curr. Opin. Virol.* **2016**, *18*, 1–8. [CrossRef]

71. Ryckman, B.J.; Jarvis, M.A.; Drummond, D.D.; Nelson, J.A.; Johnson, D.C. Human cytomegalovirus entry into epithelial and endothelial cells depends on genes UL128 to UL150 and occurs by endocytosis and low-pH fusion. *J. Virol.* **2006**, *80*, 710–722. [CrossRef]
72. Noriega, V.M.; Gardner, T.J.; Redmann, V.; Bongers, G.; Lira, S.A.; Tortorella, D. Human cytomegalovirus US28 facilitates cell-to-cell viral dissemination. *Viruses* **2014**, *6*, 1202–1218. [CrossRef]
73. Grefte, A.; Harmsen, M.C.; van der Giessen, M.; Knollema, S.; van Son, W.J.; The, T.H. Presence of human cytomegalovirus (HCMV) immediate early mRNA but not ppUL83 (lower matrix protein pp65) mRNA in polymorphonuclear and mononuclear leukocytes during active HCMV infection. *J. Gen. Virol.* **1994**, *75*, 1989–1998. [CrossRef]
74. Gerna, G.; Zipeto, D.; Percivalle, E.; Parea, M.; Revello, M.G.; Maccario, R.; Peri, G.; Milanesi, G. Human cytomegalovirus infection of the major leukocyte subpopulations and evidence for initial viral replication in polymorphonuclear leukocytes from viremic patients. *J. Infect. Dis.* **1992**, *166*, 1236–1244. [CrossRef]
75. Sinzger, C.; Jahn, G. Human cytomegalovirus cell tropism and pathogenesis. *Intervirology* **1996**, *39*, 302–319. [CrossRef] [PubMed]
76. Taylor-Wiedeman, J.; Sissons, P.; Sinclair, J. Induction of endogenous human cytomegalovirus gene expression after differentiation of monocytes from healthy carriers. *J. Virol.* **1994**, *68*, 1597–1604. [CrossRef] [PubMed]
77. Gerna, G.; Percivalle, E.; Baldanti, F.; Sozzani, S.; Lanzarini, P.; Genini, E.; Lilleri, D.; Revello, M.G. Human cytomegalovirus replicates abortively in polymorphonuclear leukocytes after transfer from infected endothelial cells via transient microfusion events. *J. Virol.* **2000**, *74*, 5629–5638. [CrossRef] [PubMed]
78. Dogra, P.; Miller-Kittrell, M.; Pitt, E.; Jackson, J.W.; Masi, T.; Copeland, C.; Wu, S.; Miller, W.E.; Sparer, T. A little cooperation helps murine cytomegalovirus (MCMV) go a long way: MCMV co-infection rescues a chemokine salivary gland defect. *J. Gen. Virol.* **2016**, *97*, 2957–2972. [CrossRef]
79. Selgrade, M.K.; Osborn, J.E. Role of macrophages in resistance to murine cytomegalovirus. *Infect. Immun.* **1974**, *10*, 1383–1390. [CrossRef]
80. Farrell, H.E.; Davis-Poynter, N.; Bruce, K.; Lawler, C.; Dolken, L.; Mach, M.; Stevenson, P.G. Lymph Node Macrophages Restrict Murine Cytomegalovirus Dissemination. *J. Virol.* **2015**, *89*, 7147–7158. [CrossRef]
81. Heo, J.; Dogra, P.; Masi, T.J.; Pitt, E.A.; de Kruijf, P.; Smit, M.J.; Sparer, T.E. Novel Human Cytomegalovirus Viral Chemokines, vCXCL-1s, Display Functional Selectivity for Neutrophil Signaling and Function. *J. Immunol.* **2015**, *195*, 227–236. [CrossRef]
82. Penfold, M.E.; Dairaghi, D.J.; Duke, G.M.; Saederup, N.; Mocarski, E.S.; Kemble, G.W.; Schall, T.J. Cytomegalovirus encodes a potent alpha chemokine. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 9839–9844. [CrossRef]
83. Yamin, R.; Lecker, L.S.M.; Weisblum, Y.; Vitenshtein, A.; Le-Trilling, V.T.K.; Wolf, D.G.; Mandelboim, O. HCMV vCXCL1 Binds Several Chemokine Receptors and Preferentially Attracts Neutrophils over NK Cells by Interacting with CXCR2. *Cell Rep.* **2016**, *15*, 1542–1553. [CrossRef]
84. Hassan-Walker, A.F.; Okwuadi, S.; Lee, L.; Griffiths, P.D.; Emery, V.C. Sequence variability of the alpha-chemokine UL146 from clinical strains of human cytomegalovirus. *J. Med. Virol.* **2004**, *74*, 573–579. [CrossRef]
85. Zheng, Q.; Tao, R.; Gao, H.; Xu, J.; Shang, S.; Zhao, N. HCMV-encoded UL128 enhances TNF-α and IL-6 expression and promotes PBMC proliferation through the MAPK/ERK pathway in vitro. *Viral. Immunol.* **2012**, *25*, 98–105. [CrossRef] [PubMed]
86. Saederup, N.; Lin, Y.C.; Dairaghi, D.J.; Schall, T.J.; Mocarski, E.S. Cytomegalovirus-encoded beta chemokine promotes monocyte-associated viremia in the host. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 10881–10886. [CrossRef]
87. Saederup, N.; Aguirre, S.A.; Sparer, T.E.; Bouley, D.M.; Mocarski, E.S. Murine cytomegalovirus CC chemokine homolog MCK-2 (m131-129) is a determinant of dissemination that increases inflammation at initial sites of infection. *J. Virol.* **2001**, *75*, 9966–9976. [CrossRef]
88. Ibanez, C.E.; Schrier, R.; Ghazal, P.; Wiley, C.; Nelson, J.A. Human cytomegalovirus productively infects primary differentiated macrophages. *J. Virol.* **1991**, *65*, 6581–6588. [CrossRef] [PubMed]
89. Lathey, J.L.; Spector, S.A. Unrestricted replication of human cytomegalovirus in hydrocortisone-treated macrophages. *J. Virol.* **1991**, *65*, 6371–6375. [CrossRef] [PubMed]

90. Waldman, W.J.; Knight, D.A.; Huang, E.H.; Sedmak, D.D. Bidirectional transmission of infectious cytomegalovirus between monocytes and vascular endothelial cells: An in vitro model. *J. Infect. Dis.* **1995**, *171*, 263–272. [CrossRef]
91. Bentz, G.L.; Jarquin-Pardo, M.; Chan, G.; Smith, M.S.; Sinzger, C.; Yurochko, A.D. Human cytomegalovirus (HCMV) infection of endothelial cells promotes naive monocyte extravasation and transfer of productive virus to enhance hematogenous dissemination of HCMV. *J. Virol.* **2006**, *80*, 11539–11555. [CrossRef]
92. Gerna, G.; Baldanti, F.; Revello, M.G. Pathogenesis of human cytomegalovirus infection and cellular targets. *Hum. Immunol.* **2004**, *65*, 381–386. [CrossRef]
93. Théry, C.; Amigorena, S. The cell biology of antigen presentation in dendritic cells. *Curr. Opin. Immunol.* **2001**, *13*, 45–51. [CrossRef]
94. Shenk, T.E.; Stinski, M.F. Human cytomegalovirus. In *Current Topics in Microbiology and Immunology*; Springer: Berlin/Heidelberg, Germany, 2008; ISBN 978-3-540-77348-1.
95. Sinclair, J.H.; Reeves, M.B. Human cytomegalovirus manipulation of latently infected cells. *Viruses* **2013**, *5*, 2803–2824. [CrossRef]
96. Sinclair, J. Manipulation of dendritic cell functions by human cytomegalovirus. *Expert Rev. Mol. Med.* **2008**, *10*, e35. [CrossRef] [PubMed]
97. Sinclair, J. Human cytomegalovirus: Latency and reactivation in the myeloid lineage. *J. Clin. Virol.* **2008**, *41*, 180–185. [CrossRef] [PubMed]
98. Yeager, A.S. Transfusion-acquired cytomegalovirus infection in newborn infants. *Am. J. Dis. Child.* **1974**, *128*, 478–483. [CrossRef] [PubMed]
99. Adler, S.P. Transfusion-associated cytomegalovirus infections. *Rev. Infect. Dis.* **1983**, *5*, 977–993. [CrossRef] [PubMed]
100. Tolpin, M.D.; Stewart, J.A.; Warren, D.; Mojica, B.A.; Collins, M.A.; Doveikis, S.A.; Cabradilla, C.; Schauf, V.; Raju, T.N.; Nelson, K. Transfusion transmission of cytomegalovirus confirmed by restriction endonuclease analysis. *J. Pediatr.* **1985**, *107*, 953–956. [CrossRef]
101. Thiele, T.; Krüger, W.; Zimmermann, K.; Ittermann, T.; Wessel, A.; Steinmetz, I.; Dölken, G.; Greinacher, A. Transmission of cytomegalovirus (CMV) infection by leukoreduced blood products not tested for CMV antibodies: A single-center prospective study in high-risk patients undergoing allogeneic hematopoietic stem cell transplantation (CME). *Transfusion* **2011**, *51*, 2620–2626. [CrossRef]
102. Taylor-Wiedeman, J.; Sissons, J.G.; Borysiewicz, L.K.; Sinclair, J.H. Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells. *J. Gen. Virol.* **1991**, *72*, 2059–2064. [CrossRef]
103. Mendelson, M.; Monard, S.; Sissons, P.; Sinclair, J. Detection of endogenous human cytomegalovirus in $CD34^+$ bone marrow progenitors. *J. Gen. Virol.* **1996**, *77*, 3099–3102. [CrossRef]
104. Reeves, M.B.; MacAry, P.A.; Lehner, P.J.; Sissons, J.G.P.; Sinclair, J.H. Latency, chromatin remodeling, and reactivation of human cytomegalovirus in the dendritic cells of healthy carriers. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 4140–4145. [CrossRef]
105. Slobedman, B.; Mocarski, E.S. Quantitative analysis of latent human cytomegalovirus. *J. Virol.* **1999**, *73*, 4806–4812. [CrossRef]
106. Saffert, R.T.; Kalejta, R.F. Human cytomegalovirus gene expression is silenced by Daxx-mediated intrinsic immune defense in model latent infections established in vitro. *J. Virol.* **2007**, *81*, 9109–9120. [CrossRef] [PubMed]
107. Yee, L.-F.; Lin, P.L.; Stinski, M.F. Ectopic expression of HCMV IE72 and IE86 proteins is sufficient to induce early gene expression but not production of infectious virus in undifferentiated promonocytic THP-1 cells. *Virology* **2007**, *363*, 174–188. [CrossRef] [PubMed]
108. O'Connor, C.M.; Murphy, E.A. A Myeloid Progenitor Cell Line Capable of Supporting Human Cytomegalovirus Latency and Reactivation, Resulting in Infectious Progeny. *J. Virol.* **2012**, *86*, 9854–9865. [CrossRef] [PubMed]
109. Penkert, R.R.; Kalejta, R.F. Human embryonic stem cell lines model experimental human cytomegalovirus latency. *MBio* **2013**, *4*, e00298-13. [CrossRef]
110. Poole, E.; McGregor Dallas, S.R.; Colston, J.; Joseph, R.S.V.; Sinclair, J. Virally induced changes in cellular microRNAs maintain latency of human cytomegalovirus in $CD34^+$ progenitors. *J. Gen. Virol.* **2011**, *92*, 1539–1549. [CrossRef]

111. Reeves, M.B.; Coleman, H.; Chadderton, J.; Goddard, M.; Sissons, J.G.P.; Sinclair, J.H. Vascular endothelial and smooth muscle cells are unlikely to be major sites of latency of human cytomegalovirus in vivo. *J. Gen. Virol.* **2004**, *85*, 3337–3341. [CrossRef]
112. Kondo, K.; Kaneshima, H.; Mocarski, E.S. Human cytomegalovirus latent infection of granulocyte-macrophage progenitors. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 11879–11883. [CrossRef]
113. Avdic, S.; Cao, J.Z.; Cheung, A.K.L.; Abendroth, A.; Slobedman, B. Viral interleukin-10 expressed by human cytomegalovirus during the latent phase of infection modulates latently infected myeloid cell differentiation. *J. Virol.* **2011**, *85*, 7465–7471. [CrossRef]
114. Bego, M.; Maciejewski, J.; Khaiboullina, S.; Pari, G.; St. Jeor, S. Characterization of an Antisense Transcript Spanning the UL81-82 Locus of Human Cytomegalovirus. *J. Virol.* **2005**, *79*, 11022–11034. [CrossRef]
115. Goodrum, F.; Reeves, M.; Sinclair, J.; High, K.; Shenk, T. Human cytomegalovirus sequences expressed in latently infected individuals promote a latent infection in vitro. *Blood* **2007**, *110*, 937–945. [CrossRef]
116. Hargett, D.; Shenk, T.E. Experimental human cytomegalovirus latency in CD14+ monocytes. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 20039–20044. [CrossRef] [PubMed]
117. Elder, E.; Sinclair, J. HCMV latency: What regulates the regulators? *Med. Microbiol. Immunol.* **2019**, *208*, 431–438. [CrossRef] [PubMed]
118. Murphy, J.C.; Fischle, W.; Verdin, E.; Sinclair, J.H. Control of cytomegalovirus lytic gene expression by histone acetylation. *EMBO J.* **2002**, *21*, 1112–1120. [CrossRef] [PubMed]
119. Reeves, M.B.; Lehner, P.J.; Sissons, J.G.P.; Sinclair, J.H. An in vitro model for the regulation of human cytomegalovirus latency and reactivation in dendritic cells by chromatin remodelling. *J. Gen. Virol.* **2005**, *86*, 2949–2954. [CrossRef] [PubMed]
120. Schwartz, M.; Stern-Ginossar, N. The Transcriptome of Latent Human Cytomegalovirus. *J. Virol.* **2019**, *93*. [CrossRef]
121. Rossetto, C.C.; Tarrant-Elorza, M.; Pari, G.S. Cis and trans acting factors involved in human cytomegalovirus experimental and natural latent infection of CD14 (+) monocytes and CD34 (+) cells. *PLoS Pathog.* **2013**, *9*, e1003366. [CrossRef]
122. Margueron, R.; Reinberg, D. The Polycomb complex PRC2 and its mark in life. *Nature* **2011**, *469*, 343–349. [CrossRef]
123. Stark, T.J.; Arnold, J.D.; Spector, D.H.; Yeo, G.W. High-resolution profiling and analysis of viral and host small RNAs during human cytomegalovirus infection. *J. Virol.* **2012**, *86*, 226–235. [CrossRef]
124. Meshesha, M.K.; Bentwich, Z.; Solomon, S.A.; Avni, Y.S. In vivo expression of human cytomegalovirus (HCMV) microRNAs during latency. *Gene* **2016**, *575*, 101–107. [CrossRef]
125. Pan, C.; Zhu, D.; Wang, Y.; Li, L.; Li, D.; Liu, F.; Zhang, C.-Y.; Zen, K. Human Cytomegalovirus miR-UL148D Facilitates Latent Viral Infection by Targeting Host Cell Immediate Early Response Gene 5. *PLoS Pathog.* **2016**, *12*, e1006007. [CrossRef]
126. Lau, B.; Poole, E.; Krishna, B.; Sellart, I.; Wills, M.R.; Murphy, E.; Sinclair, J. The Expression of Human Cytomegalovirus MicroRNA MiR-UL148D during Latent Infection in Primary Myeloid Cells Inhibits Activin A-triggered Secretion of IL-6. *Sci Rep* **2016**, *6*, 31205. [CrossRef] [PubMed]
127. Diggins, N.L.; Hancock, M.H. HCMV miRNA Targets Reveal Important Cellular Pathways for Viral Replication, Latency, and Reactivation. *Noncoding RNA* **2018**, *4*, 29. [CrossRef] [PubMed]
128. Kim, Y.; Lee, S.; Kim, S.; Kim, D.; Ahn, J.-H.; Ahn, K. Human cytomegalovirus clinical strain-specific microRNA miR-UL148D targets the human chemokine RANTES during infection. *PLoS Pathog.* **2012**, *8*, e1002577. [CrossRef] [PubMed]
129. Fu, M.; Gao, Y.; Zhou, Q.; Zhang, Q.; Peng, Y.; Tian, K.; Wang, J.; Zheng, X. Human cytomegalovirus latent infection alters the expression of cellular and viral microRNA. *Gene* **2014**, *536*, 272–278. [CrossRef]
130. Beisser, P.S.; Laurent, L.; Virelizier, J.L.; Michelson, S. Human cytomegalovirus chemokine receptor gene US28 is transcribed in latently infected THP-1 monocytes. *J. Virol.* **2001**, *75*, 5949–5957. [CrossRef]
131. Cheung, A.K.L.; Gottlieb, D.J.; Plachter, B.; Pepperl-Klindworth, S.; Avdic, S.; Cunningham, A.L.; Abendroth, A.; Slobedman, B. The role of the human cytomegalovirus UL111A gene in down-regulating CD4+ T-cell recognition of latently infected cells: Implications for virus elimination during latency. *Blood* **2009**, *114*, 4128–4137. [CrossRef]

132. Cheung, T.C.; Humphreys, I.R.; Potter, K.G.; Norris, P.S.; Shumway, H.M.; Tran, B.R.; Patterson, G.; Jean-Jacques, R.; Yoon, M.; Spear, P.G.; et al. Evolutionarily divergent herpesviruses modulate T cell activation by targeting the herpesvirus entry mediator cosignaling pathway. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 13218–13223. [CrossRef]
133. Poole, E.; Groves, I.; MacDonald, A.; Pang, Y.; Alcami, A.; Sinclair, J. Identification of TRIM23 as a cofactor involved in the regulation of NF-kappaB by human cytomegalovirus. *J. Virol.* **2009**, *83*, 3581–3590. [CrossRef]
134. Keyes, L.R.; Hargett, D.; Soland, M.; Bego, M.G.; Rossetto, C.C.; Almeida-Porada, G.; St Jeor, S. HCMV protein LUNA is required for viral reactivation from latently infected primary $CD14^+$ cells. *PLoS ONE* **2012**, *7*, e52827. [CrossRef]
135. Weekes, M.P.; Tan, S.Y.L.; Poole, E.; Talbot, S.; Antrobus, R.; Smith, D.L.; Montag, C.; Gygi, S.P.; Sinclair, J.H.; Lehner, P.J. Latency-associated degradation of the MRP1 drug transporter during latent human cytomegalovirus infection. *Science* **2013**, *340*, 199–202. [CrossRef]
136. Hahn, G.; Jores, R.; Mocarski, E.S. Cytomegalovirus remains latent in a common precursor of dendritic and myeloid cells. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 3937–3942. [CrossRef] [PubMed]
137. Reeves, M.B.; Compton, T. Inhibition of inflammatory interleukin-6 activity via extracellular signal-regulated kinase-mitogen-activated protein kinase signaling antagonizes human cytomegalovirus reactivation from dendritic cells. *J. Virol.* **2011**, *85*, 12750–12758. [CrossRef]
138. Söderberg-Nauclér, C.; Fish, K.N.; Nelson, J.A. Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. *Cell* **1997**, *91*, 119–126. [CrossRef]
139. Humar, A.; St Louis, P.; Mazzulli, T.; McGeer, A.; Lipton, J.; Messner, H.; MacDonald, K.S. Elevated serum cytokines are associated with cytomegalovirus infection and disease in bone marrow transplant recipients. *J. Infect. Dis.* **1999**, *179*, 484–488. [CrossRef] [PubMed]
140. Tong, C.Y.; Bakran, A.; Williams, H.; Cuevas, L.E.; Peiris, J.S.; Hart, C.A. Association of tumour necrosis factor alpha and interleukin 6 levels with cytomegalovirus DNA detection and disease after renal transplantation. *J. Med. Virol.* **2001**, *64*, 29–34. [CrossRef] [PubMed]
141. Smith, M.G. Propagation of salivary gland virus of the mouse in tissue cultures. *Proc. Soc. Exp. Biol. Med.* **1954**, *86*, 435–440. [CrossRef]
142. Craig, J.M.; Macauley, J.C.; Weller, T.H.; Wirth, P. Isolation of intranuclear inclusion producing agents from infants with illnesses resembling cytomegalic inclusion disease. *Proc. Soc. Exp. Biol. Med.* **1957**, *94*, 4–12. [CrossRef]
143. Chee, M.S.; Bankier, A.T.; Beck, S.; Bohni, R.; Brown, C.M.; Cerny, R.; Horsnell, T.; Hutchison, C.A.; Kouzarides, T.; Martignetti, J.A. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Curr. Top. Microbiol. Immunol.* **1990**, *154*, 125–169. [CrossRef]
144. Neff, B.J.; Weibel, R.E.; Buynak, E.B.; McLean, A.A.; Hilleman, M.R. Clinical and laboratory studies of live cytomegalovirus vaccine Ad-169. *Proc. Soc. Exp. Biol. Med.* **1979**, *160*, 32–37. [CrossRef]
145. Plotkin, S.A.; Farquhar, J.; Horberger, E. Clinical trials of immunization with the Towne 125 strain of human cytomegalovirus. *J. Infect. Dis.* **1976**, *134*, 470–475. [CrossRef]
146. Quinnan, G.V.; Delery, M.; Rook, A.H.; Frederick, W.R.; Epstein, J.S.; Manischewitz, J.F.; Jackson, L.; Ramsey, K.M.; Mittal, K.; Plotkin, S.A. Comparative virulence and immunogenicity of the Towne strain and a nonattenuated strain of cytomegalovirus. *Ann. Intern. Med.* **1984**, *101*, 478–483. [CrossRef]
147. Cha, T.A.; Tom, E.; Kemble, G.W.; Duke, G.M.; Mocarski, E.S.; Spaete, R.R. Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains. *J. Virol.* **1996**, *70*, 78–83. [CrossRef] [PubMed]
148. Prichard, M.N.; Penfold, M.E.; Duke, G.M.; Spaete, R.R.; Kemble, G.W. A review of genetic differences between limited and extensively passaged human cytomegalovirus strains. *Rev. Med. Virol.* **2001**, *11*, 191–200. [CrossRef] [PubMed]
149. Dargan, D.J.; Douglas, E.; Cunningham, C.; Jamieson, F.; Stanton, R.J.; Baluchova, K.; McSharry, B.P.; Tomasec, P.; Emery, V.C.; Percivalle, E.; et al. Sequential mutations associated with adaptation of human cytomegalovirus to growth in cell culture. *J. Gen. Virol.* **2010**, *91*, 1535–1546. [CrossRef] [PubMed]
150. Wilkinson, G.W.G.; Davison, A.J.; Tomasec, P.; Fielding, C.A.; Aicheler, R.; Murrell, I.; Seirafian, S.; Wang, E.C.Y.; Weekes, M.; Lehner, P.J.; et al. Human cytomegalovirus: Taking the strain. *Med. Microbiol. Immunol.* **2015**, *204*, 273–284. [CrossRef]

151. Bradley, A.J.; Lurain, N.S.; Ghazal, P.; Trivedi, U.; Cunningham, C.; Baluchova, K.; Gatherer, D.; Wilkinson, G.W.G.; Dargan, D.J.; Davison, A.J. High-throughput sequence analysis of variants of human cytomegalovirus strains Towne and AD169. *J. Gen. Virol.* **2009**, *90*, 2375–2380. [CrossRef]
152. Jung, G.S.; Kim, Y.Y.; Kim, J.I.; Ji, G.Y.; Jeon, J.S.; Yoon, H.W.; Lee, G.-C.; Ahn, J.H.; Lee, K.M.; Lee, C.H. Full genome sequencing and analysis of human cytomegalovirus strain JHC isolated from a Korean patient. *Virus Res.* **2011**, *156*, 113–120. [CrossRef]
153. Sijmons, S.; Thys, K.; Mbong Ngwese, M.; Van Damme, E.; Dvorak, J.; Van Loock, M.; Li, G.; Tachezy, R.; Busson, L.; Aerssens, J.; et al. High-throughput analysis of human cytomegalovirus genome diversity highlights the widespread occurrence of gene-disrupting mutations and pervasive recombination. *J. Virol.* **2015**, *89*, 7673–7695. [CrossRef]
154. Dunn, W.; Chou, C.; Li, H.; Hai, R.; Patterson, D.; Stolc, V.; Zhu, H.; Liu, F. Functional profiling of a human cytomegalovirus genome. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 14223–14228. [CrossRef]
155. Murphy, E.; Yu, D.; Grimwood, J.; Schmutz, J.; Dickson, M.; Jarvis, M.A.; Hahn, G.; Nelson, J.A.; Myers, R.M.; Shenk, T.E. Coding potential of laboratory and clinical strains of human cytomegalovirus. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 14976–14981. [CrossRef]
156. Sinzger, C.; Hahn, G.; Digel, M.; Katona, R.; Sampaio, K.L.; Messerle, M.; Hengel, H.; Koszinowski, U.; Brune, W.; Adler, B. Cloning and sequencing of a highly productive, endotheliotropic virus strain derived from human cytomegalovirus TB40/E. *J. Gen. Virol.* **2008**, *89*, 359–368. [CrossRef] [PubMed]
157. Dolan, A.; Cunningham, C.; Hector, R.D.; Hassan-Walker, A.F.; Lee, L.; Addison, C.; Dargan, D.J.; McGeoch, D.J.; Gatherer, D.; Emery, V.C.; et al. Genetic content of wild-type human cytomegalovirus. *J. Gen. Virol.* **2004**, *85*, 1301–1312. [CrossRef] [PubMed]
158. Virus Pathogen Database and Analysis Resource (ViPR)—Genome Database with Visualization and Analysis Tools. Available online: https://www.viprbrc.org/brc/home.spg?decorator=vipr (accessed on 23 April 2020).
159. Pickett, B.E.; Sadat, E.L.; Zhang, Y.; Noronha, J.M.; Squires, R.B.; Hunt, V.; Liu, M.; Kumar, S.; Zaremba, S.; Gu, Z.; et al. ViPR: An open bioinformatics database and analysis resource for virology research. *Nucleic Acids Res.* **2012**, *40*, D593–D598. [CrossRef] [PubMed]
160. Renzette, N.; Bhattacharjee, B.; Jensen, J.D.; Gibson, L.; Kowalik, T.F. Extensive genome-wide variability of human cytomegalovirus in congenitally infected infants. *PLoS Pathog.* **2011**, *7*, e1001344. [CrossRef] [PubMed]
161. Renzette, N.; Gibson, L.; Jensen, J.D.; Kowalik, T.F. Human cytomegalovirus intrahost evolution-a new avenue for understanding and controlling herpesvirus infections. *Curr. Opin. Virol.* **2014**, *8*, 109–115. [CrossRef] [PubMed]
162. Renzette, N.; Pfeifer, S.P.; Matuszewski, S.; Kowalik, T.F.; Jensen, J.D. On the Analysis of Intrahost and Interhost Viral Populations: Human Cytomegalovirus as a Case Study of Pitfalls and Expectations. *J. Virol.* **2017**, *91*. [CrossRef]
163. Peck, K.M.; Lauring, A.S. Complexities of Viral Mutation Rates. *J. Virol.* **2018**, *92*. [CrossRef]
164. Vabret, N.; Bhardwaj, N.; Greenbaum, B.D. Sequence-Specific Sensing of Nucleic Acids. *Trends Immunol.* **2017**, *38*, 53–65. [CrossRef]
165. Christensen, M.H.; Paludan, S.R. Viral evasion of DNA-stimulated innate immune responses. *Cell. Mol. Immunol.* **2017**, *14*, 4–13. [CrossRef]
166. Hage, E.; Wilkie, G.S.; Linnenweber-Held, S.; Dhingra, A.; Suárez, N.M.; Schmidt, J.J.; Kay-Fedorov, P.C.; Mischak-Weissinger, E.; Heim, A.; Schwarz, A.; et al. Characterization of Human Cytomegalovirus Genome Diversity in Immunocompromised Hosts by Whole-Genome Sequencing Directly From Clinical Specimens. *J. Infect. Dis.* **2017**, *215*, 1673–1683. [CrossRef]
167. Suárez, N.M.; Wilkie, G.S.; Hage, E.; Camiolo, S.; Holton, M.; Hughes, J.; Maabar, M.; Vattipally, S.B.; Dhingra, A.; Gompels, U.A.; et al. Human Cytomegalovirus Genomes Sequenced Directly From Clinical Material: Variation, Multiple-Strain Infection, Recombination, and Gene Loss. *J. Infect. Dis.* **2019**, *220*, 781–791. [CrossRef] [PubMed]
168. Cudini, J.; Roy, S.; Houldcroft, C.J.; Bryant, J.M.; Depledge, D.P.; Tutill, H.; Veys, P.; Williams, R.; Worth, A.J.J.; Tamuri, A.U.; et al. Human cytomegalovirus haplotype reconstruction reveals high diversity due to superinfection and evidence of within-host recombination. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 5693–5698. [CrossRef] [PubMed]

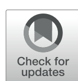
169. Lassalle, F.; Depledge, D.P.; Reeves, M.B.; Brown, A.C.; Christiansen, M.T.; Tutill, H.J.; Williams, R.J.; Einer-Jensen, K.; Holdstock, J.; Atkinson, C.; et al. Islands of linkage in an ocean of pervasive recombination reveals two-speed evolution of human cytomegalovirus genomes. *Virus Evol.* **2016**, *2*, vew017. [CrossRef] [PubMed]
170. Houldcroft, C.J.; Cudini, J.; Goldstein, R.A.; Breuer, J. Reply to Jensen and Kowalik: Consideration of mixed infections is central to understanding HCMV intrahost diversity. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 818–819. [CrossRef] [PubMed]
171. Jensen, J.D.; Kowalik, T.F. A consideration of within-host human cytomegalovirus genetic variation. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 816–817. [CrossRef]
172. Paradowska, E.; Jabłońska, A.; Studzińska, M.; Kasztelewicz, B.; Zawilińska, B.; Wiśniewska-Ligier, M.; Dzierżanowska-Fangrat, K.; Woźniakowska-Gęsicka, T.; Kosz-Vnenchak, M.; Leśnikowski, Z.J. Cytomegalovirus glycoprotein H genotype distribution and the relationship with hearing loss in children. *J. Med. Virol.* **2014**, *86*, 1421–1427. [CrossRef]
173. Bradley, A.J.; Kovács, I.J.; Gatherer, D.; Dargan, D.J.; Alkharsah, K.R.; Chan, P.K.S.; Carman, W.F.; Dedicoat, M.; Emery, V.C.; Geddes, C.C.; et al. Genotypic analysis of two hypervariable human cytomegalovirus genes. *J. Med. Virol.* **2008**, *80*, 1615–1623. [CrossRef]
174. Stratton, K.R.; Durch, J.S.; Lawrence, R.S. *Vaccines for the 21st Century: A Tool for Decisionmaking*; Institute of Medicine (US) Committee to Study Priorities for Vaccine Development; National Academies Press: Washington, DC, USA, 2000; ISBN 978-0-309-05646-5.
175. Picone, O.; Vauloup-Fellous, C.; Cordier, A.G.; Guitton, S.; Senat, M.V.; Fuchs, F.; Ayoubi, J.M.; Grangeot Keros, L.; Benachi, A. A series of 238 cytomegalovirus primary infections during pregnancy: Description and outcome. *Prenat. Diagn.* **2013**, *33*, 751–758. [CrossRef]
176. Fouts, A.E.; Chan, P.; Stephan, J.-P.; Vandlen, R.; Feierbach, B. Antibodies against the gH/gL/UL128/UL130/UL131 complex comprise the majority of the anti-cytomegalovirus (anti-CMV) neutralizing antibody response in CMV hyperimmune globulin. *J. Virol.* **2012**, *86*, 7444–7447. [CrossRef]
177. Elek, S.D.; Stern, H. Development of a vaccine against mental retardation caused by cytomegalovirus infection in utero. *Lancet* **1974**, *1*, 1–5. [CrossRef]
178. Plotkin, S.A.; Furukawa, T.; Zygraich, N.; Huygelen, C. Candidate cytomegalovirus strain for human vaccination. *Infect. Immun.* **1975**, *12*, 521–527. [CrossRef] [PubMed]
179. Plotkin, S.A. Is there a formula for an effective CMV vaccine? *J. Clin. Virol.* **2002**, *25*, S13–S21. [CrossRef]
180. Wang, D.; Shenk, T. Human cytomegalovirus virion protein complex required for epithelial and endothelial cell tropism. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 18153–18158. [CrossRef] [PubMed]
181. Cui, X.; Meza, B.P.; Adler, S.P.; McVoy, M.A. Cytomegalovirus vaccines fail to induce epithelial entry neutralizing antibodies comparable to natural infection. *Vaccine* **2008**, *26*, 5760–5766. [CrossRef] [PubMed]
182. Adler, S.P.; Manganello, A.-M.; Lee, R.; McVoy, M.A.; Nixon, D.E.; Plotkin, S.; Mocarski, E.; Cox, J.H.; Fast, P.E.; Nesterenko, P.A.; et al. A Phase 1 Study of 4 Live, Recombinant Human Cytomegalovirus Towne/Toledo Chimera Vaccines in Cytomegalovirus–Seronegative Men. *J. Infect. Dis.* **2016**, *214*, 1341–1348. [CrossRef]
183. Wang, D.; Freed, D.C.; He, X.; Li, F.; Tang, A.; Cox, K.S.; Dubey, S.A.; Cole, S.; Medi, M.B.; Liu, Y.; et al. A replication-defective human cytomegalovirus vaccine for prevention of congenital infection. *Sci. Transl. Med.* **2016**, *8*, 362ra145. [CrossRef]
184. Liu, Y.; Freed, D.C.; Li, L.; Tang, A.; Li, F.; Murray, E.M.; Adler, S.P.; McVoy, M.A.; Rupp, R.E.; Barrett, D.; et al. A Replication-Defective Human Cytomegalovirus Vaccine Elicits Humoral Immune Responses Analogous to Those with Natural Infection. *J. Virol.* **2019**, *93*. [CrossRef]
185. Gerna, G.; Lilleri, D. Human cytomegalovirus (HCMV) infection/re-infection: Development of a protective HCMV vaccine. *New Microbiol.* **2019**, *42*, 1–20.
186. Gonczol, E.; Ianacone, J.; Ho, W.Z.; Starr, S.; Meignier, B.; Plotkin, S. Isolated gA/gB glycoprotein complex of human cytomegalovirus envelope induces humoral and cellular immune-responses in human volunteers. *Vaccine* **1990**, *8*, 130–136. [CrossRef]
187. Mitchell, D.K.; Holmes, S.J.; Burke, R.L.; Duliege, A.M.; Adler, S.P. Immunogenicity of a recombinant human cytomegalovirus gB vaccine in seronegative toddlers. *Pediatr. Infect. Dis. J.* **2002**, *21*, 133–138. [CrossRef]
188. Selinsky, C.; Luke, C.; Wloch, M.; Geall, A.; Hermanson, G.; Kaslow, D.; Evans, T. A DNA-based vaccine for the prevention of human cytomegalovirus-associated diseases. *Hum. Vaccin.* **2005**, *1*, 16–23. [CrossRef] [PubMed]

189. John, S.; Yuzhakov, O.; Woods, A.; Deterling, J.; Hassett, K.; Shaw, C.A.; Ciaramella, G. Multi-antigenic human cytomegalovirus mRNA vaccines that elicit potent humoral and cell-mediated immunity. *Vaccine* **2018**, *36*, 1689–1699. [CrossRef] [PubMed]
190. Nelson, C.S.; Jenks, J.A.; Pardi, N.; Goodwin, M.; Roark, H.; Edwards, W.; McLellan, J.S.; Pollara, J.; Weissman, D.; Permar, S.R. Human Cytomegalovirus Glycoprotein B Nucleoside-Modified mRNA Vaccine Elicits Antibody Responses with Greater Durability and Breadth than MF59-Adjuvanted gB Protein Immunization. *J. Virol.* **2020**, *94*. [CrossRef] [PubMed]
191. La Rosa, C.; Longmate, J.; Lacey, S.F.; Kaltcheva, T.; Sharan, R.; Marsano, D.; Kwon, P.; Drake, J.; Williams, B.; Denison, S.; et al. Clinical evaluation of safety and immunogenicity of PADRE-cytomegalovirus (CMV) and tetanus-CMV fusion peptide vaccines with or without PF03512676 adjuvant. *J. Infect. Dis.* **2012**, *205*, 1294–1304. [CrossRef] [PubMed]
192. Kirchmeier, M.; Fluckiger, A.-C.; Soare, C.; Bozic, J.; Ontsouka, B.; Ahmed, T.; Diress, A.; Pereira, L.; Schödel, F.; Plotkin, S.; et al. Enveloped virus-like particle expression of human cytomegalovirus glycoprotein B antigen induces antibodies with potent and broad neutralizing activity. *Clin. Vaccine Immunol.* **2014**, *21*, 174–180. [CrossRef] [PubMed]
193. Cayatte, C.; Schneider-Ohrum, K.; Wang, Z.; Irrinki, A.; Nguyen, N.; Lu, J.; Nelson, C.; Servat, E.; Gemmell, L.; Citkowicz, A.; et al. Cytomegalovirus vaccine strain towne-derived dense bodies induce broad cellular immune responses and neutralizing antibodies that prevent infection of fibroblasts and epithelial cells. *J. Virol.* **2013**, *87*, 11107–11120. [CrossRef] [PubMed]
194. Britt, W.J.; Prichard, M.N. New therapies for human cytomegalovirus infections. *Antivir. Res.* **2018**, *159*, 153–174. [CrossRef]
195. El Helou, G.; Razonable, R.R. Safety considerations with current and emerging antiviral therapies for cytomegalovirus infection in transplantation. *Expert Opin. Drug Saf.* **2019**, *18*, 1017–1030. [CrossRef]
196. Poole, C.L.; James, S.H. Antiviral Therapies for Herpesviruses: Current Agents and New Directions. *Clin. Ther.* **2018**, *40*, 1282–1298. [CrossRef]
197. Kim, E.S. Letermovir: First Global Approval. *Drugs* **2018**, *78*, 147–152. [CrossRef]
198. Stein, C.A.; Castanotto, D. FDA-Approved Oligonucleotide Therapies in 2017. *Mol. Ther.* **2017**, *25*, 1069–1075. [CrossRef] [PubMed]
199. Kimberlin, D.W.; Jester, P.M.; Sánchez, P.J.; Ahmed, A.; Arav-Boger, R.; Michaels, M.G.; Ashouri, N.; Englund, J.A.; Estrada, B.; Jacobs, R.F.; et al. Valganciclovir for symptomatic congenital cytomegalovirus disease. *N. Engl. J. Med.* **2015**, *372*, 933–943. [CrossRef] [PubMed]
200. Kotton, C.N.; Kumar, D.; Caliendo, A.M.; Huprikar, S.; Chou, S.; Danziger-Isakov, L.; Humar, A. The Transplantation Society International CMV Consensus Group The Third International Consensus Guidelines on the Management of Cytomegalovirus in Solid-organ Transplantation. *Transplantation* **2018**, *102*, 900–931. [CrossRef] [PubMed]
201. Green, M.L.; Leisenring, W.; Xie, H.; Mast, T.C.; Cui, Y.; Sandmaier, B.M.; Sorror, M.L.; Goyal, S.; Özkök, S.; Yi, J.; et al. Cytomegalovirus viral load and mortality after haemopoietic stem cell transplantation in the era of pre-emptive therapy: A retrospective cohort study. *Lancet Haematol.* **2016**, *3*, e119–e127. [CrossRef]
202. Littler, E.; Stuart, A.D.; Chee, M.S. Human cytomegalovirus UL97 open reading frame encodes a protein that phosphorylates the antiviral nucleoside analogue ganciclovir. *Nature* **1992**, *358*, 160–162. [CrossRef]
203. Gentry, B.G.; Gentry, S.N.; Jackson, T.L.; Zemlicka, J.; Drach, J.C. Phosphorylation of antiviral and endogenous nucleotides to di- and triphosphates by guanosine monophosphate kinase. *Biochem. Pharmacol.* **2011**, *81*, 43–49. [CrossRef]
204. Chou, S. Approach to drug-resistant cytomegalovirus in transplant recipients. *Curr. Opin. Infect. Dis.* **2015**, *28*, 293–299. [CrossRef]
205. Lischka, P.; Zimmermann, H. Antiviral strategies to combat cytomegalovirus infections in transplant recipients. *Curr. Opin. Pharmacol.* **2008**, *8*, 541–548. [CrossRef]
206. De Clercq, E.; Holý, A. Acyclic nucleoside phosphonates: A key class of antiviral drugs. *Nat. Rev. Drug Discov.* **2005**, *4*, 928–940. [CrossRef]
207. Wagstaff, A.J.; Bryson, H.M. Foscarnet. A reappraisal of its antiviral activity, pharmacokinetic properties and therapeutic use in immunocompromised patients with viral infections. *Drugs* **1994**, *48*, 199–226. [CrossRef]

208. Torres-Madriz, G.; Boucher, H.W. Immunocompromised hosts: Perspectives in the treatment and prophylaxis of cytomegalovirus disease in solid-organ transplant recipients. *Clin. Infect. Dis.* **2008**, *47*, 702–711. [CrossRef] [PubMed]
209. Razonable, R.R. Drug-resistant cytomegalovirus: Clinical implications of specific mutations. *Curr. Opin. Organ Transplant.* **2018**, *23*, 388–394. [CrossRef] [PubMed]
210. Stern, A.; Papanicolaou, G.A. CMV Prevention and Treatment in Transplantation: What's New in 2019. *Curr. Infect. Dis. Rep.* **2019**, *21*, 45. [CrossRef]
211. Maertens, J.; Cordonnier, C.; Jaksch, P.; Poiré, X.; Uknis, M.; Wu, J.; Wijatyk, A.; Saliba, F.; Witzke, O.; Villano, S. Maribavir for Preemptive Treatment of Cytomegalovirus Reactivation. *N. Engl. J. Med.* **2019**, *381*, 1136–1147. [CrossRef] [PubMed]
212. Buerger, I.; Reefschlaeger, J.; Bender, W.; Eckenberg, P.; Popp, A.; Weber, O.; Graeper, S.; Klenk, H.D.; Ruebsamen-Waigmann, H.; Hallenberger, S. A novel nonnucleoside inhibitor specifically targets cytomegalovirus DNA maturation via the UL89 and UL56 gene products. *J. Virol.* **2001**, *75*, 9077–9086. [CrossRef] [PubMed]
213. Schleiss, M.R.; Bernstein, D.I.; McVoy, M.A.; Stroup, G.; Bravo, F.; Creasy, B.; McGregor, A.; Henninger, K.; Hallenberger, S. The non-nucleoside antiviral, BAY 38-4766, protects against cytomegalovirus (CMV) disease and mortality in immunocompromised guinea pigs. *Antivir. Res.* **2005**, *65*, 35–43. [CrossRef]
214. Underwood, M.R.; Ferris, R.G.; Selleseth, D.W.; Davis, M.G.; Drach, J.C.; Townsend, L.B.; Biron, K.K.; Boyd, F.L. Mechanism of action of the ribopyranoside benzimidazole GW275175X against human cytomegalovirus. *Antimicrob. Agents Chemother.* **2004**, *48*, 1647–1651. [CrossRef]
215. Kotton, C.N. Updates on antiviral drugs for cytomegalovirus prevention and treatment. *Curr. Opin. Organ Transplant.* **2019**, *24*, 469–475. [CrossRef]
216. El Helou, G.; Razonable, R.R. Letermovir for the prevention of cytomegalovirus infection and disease in transplant recipients: An evidence-based review. *Infect. Drug Resist.* **2019**, *12*, 1481–1491. [CrossRef]
217. Vaismoradi, M.; Logan, P.A.; Jordan, S.; Sletvold, H. Adverse Drug Reactions in Norway: A Systematic Review. *Pharmacy* **2019**, *7*, 102. [CrossRef]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

REVIEW
published: 15 April 2020
doi: 10.3389/fmicb.2020.00661



Tuning the Orchestra: HCMV vs. Innate Immunity

*Valentina Dell'Oste[1], Matteo Biolatti[1], Ganna Galitska[1], Gloria Griffante[1], Francesca Gugliesi[1], Selina Pasquero[1], Alessandra Zingoni[2], Cristina Cerboni[2] and Marco De Andrea[1,3]**

[1] Laboratory of Pathogenesis of Viral Infections, Department of Public Health and Pediatric Sciences, University of Turin, Turin, Italy, [2] Department of Molecular Immunology and Immunopathology, "Sapienza" University of Rome, Rome, Italy, [3] Center for Translational Research on Autoimmune and Allergic Disease – CAAD, University of Piemonte Orientale, Novara, Italy

Understanding how the innate immune system keeps human cytomegalovirus (HCMV) in check has recently become a critical issue in light of the global clinical burden of HCMV infection in newborns and immunodeficient patients. Innate immunity constitutes the first line of host defense against HCMV as it involves a complex array of cooperating effectors – e.g., inflammatory cytokines, type I interferon (IFN-I), natural killer (NK) cells, professional antigen-presenting cells (APCs) and phagocytes – all capable of disrupting HCMV replication. These factors are known to trigger a highly efficient adaptive immune response, where cellular restriction factors (RFs) play a major gatekeeping role. Unlike other innate immunity components, RFs are constitutively expressed in many cell types, ready to act before pathogen exposure. Nonetheless, the existence of a positive regulatory feedback loop between RFs and IFNs is clear evidence of an intimate cooperation between intrinsic and innate immunity. In the course of virus-host coevolution, HCMV has, however, learned how to manipulate the functions of multiple cellular players of the host innate immune response to achieve latency and persistence. Thus, HCMV acts like an orchestra conductor able to piece together and rearrange parts of a musical score (i.e., innate immunity) to obtain the best live performance (i.e., viral fitness). It is therefore unquestionable that innovative therapeutic solutions able to prevent HCMV immune evasion in congenitally infected infants and immunocompromised individuals are urgently needed. Here, we provide an up-to-date review of the mechanisms regulating the interplay between HCMV and innate immunity, focusing on the various strategies of immune escape evolved by this virus to gain a fitness advantage.

Keywords: human cytomegalovirus, innate immunity, interferon system, apoptosis, restriction factors, NK cells, antigen presenting cell (APC)

OPEN ACCESS

Edited by:
Akio Adachi, Kansai Medical University, Japan

Reviewed by:
Tatsuo Suzutani, Fukushima Medical University, Japan
Rafael Solana, Universidad de Córdoba, Spain
Sarah Elizabeth Jackson, University of Cambridge, United Kingdom

Correspondence:
Marco De Andrea
marco.deandrea@unito.it

Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *25 October 2019*
Accepted: *23 March 2020*
Published: *15 April 2020*

Citation:
Dell'Oste V, Biolatti M, Galitska G, Griffante G, Gugliesi F, Pasquero S, Zingoni A, Cerboni C and De Andrea M (2020) Tuning the Orchestra: HCMV vs. Innate Immunity. Front. Microbiol. 11:661. doi: 10.3389/fmicb.2020.00661

INTRODUCTION

The innate immune response is a fundamental defense mechanism, shielding the host from constant attacks of invading pathogens of different origin, whether they are bacterial, fungal, transposon or viral (Akira et al., 2006; Yan and Chen, 2012). Thus, for a virus, successful invasion and efficient subversion of the host immediate immune response are critical steps to achieve productive infection.

Some viruses, such as herpesviruses, have succeeded in establishing lifelong persistence in humans by evading immune surveillance (Stempel et al., 2019). For example, human cytomegalovirus (HCMV), a notorious opportunistic pantropic betaherpesvirus with a worldwide seroprevalence of 50 to > 90% in adults (Cannon et al., 2010), has the remarkable ability to manipulate and evade immune detection, literally transforming the host cellular environment into an ideal niche in which to thrive (Griffiths et al., 2015). This is achieved through sophisticated manipulations of cellular gene expression or elegant evasion strategies evolved by the virus during its long lasting co-evolution with the host (Wang et al., 2007; Loewendorf and Benedict, 2010; Rossini et al., 2012).

Even though HCMV infection is asymptomatic in immunocompetent individuals, it may lead to several life-threatening conditions in immunosuppressed subjects, such as organ and stem cell transplant recipients or AIDS patients. Furthermore, it can cause severe morbidity in congenitally infected children and elderly people (Cannon et al., 2010; Manicklal et al., 2013; Tu and Rao, 2016; Britt, 2018). Additionally, spontaneous reactivation of latent endogenous virus and/or superinfection with multiple viral strains can contribute to the overall burden and individual disease severity, as neither a vaccine nor an effective cure is currently available (Schleiss et al., 2017).

Although several viral polymerase inhibitors acting upon lytic replication (e.g., ganciclovir, cidofovir, and foscarnet) are widely used to treat HCMV infections, they are characterized by high hematopoietic toxicity and poor bioavailability, which prevents their use in pregnant women and congenitally infected newborns (Britt and Prichard, 2018). In addition, targeting latent HCMV remains an unsolved issue in patient clinical management. To make matters worse, the number of drug-resistant HCMV mutants has increased dramatically over the last decade (Piret and Boivin, 2019).

The outcome and severity of HCMV infection depends predominantly on initial virus-host interactions, occurring early upon infection, when intrinsic innate immunity comes into play to fight off the virus. As a frontline defense and earliest reaction measure, innate immunity avail itself of a complex array of effector cells and soluble factors, including pro-inflammatory cytokines and type I interferon (IFN-I), natural killer (NK) cells, professional antigen-presenting cells (APCs) and phagocytes, all operating in a fine-tuned and balanced manner (Luecke and Paludan, 2015; Patel et al., 2018).

Intrinsic cellular restriction factors (RFs) are constitutively expressed and play physiological roles in uninfected cells by cooperating with innate immune effectors, as some of them appear to be IFN-inducible, thus contributing to early host defense (Bieniasz, 2004; Duggal and Emerman, 2012).

Finally, triggered cell suicide processes (i.e., apoptosis and pyroptosis), resulting in death and removal of HCMV-infected cells, can also have a major impact on viral infection progression (Brune and Andoniou, 2017).

Ultimately, the orchestra formed by these innate immune components fine-tunes a highly efficient adaptive immune response that keeps HCMV infection at bay. However, HCMV often becomes the conductor of this orchestra, and as such it can manipulate to its liking all the various components of the immune response to make the cellular environment more permissible to viral replication and survival, thereby achieving persistence, latency and ultimately seroprevalence.

HCMV has an extremely large genome, and its enhanced encoding capacity allows for generating multiple viral proteins, involved in modulation and subversion of multiple signaling pathways (Stern-Ginossar et al., 2012; Brune and Andoniou, 2017). The exact mechanisms of action and role of this large number of viral proteins have not been yet completely elucidated, although many of them are probably involved in immune evasion.

In this regard, the fact that HCMV has developed a number of ingenious strategies directed against NK cells and APCs underscores the overall importance of these cells in innate immunity. For example, NK cells can release cytotoxic granules triggered by natural or antibody-dependent cytotoxicity (ADCC) or produce cytokines upon engagement of activating and inhibitory NK cell receptors. Even though NK cells are the major cytotoxic arm of innate immunity, their contribution in shaping T cell-mediated immune responses and generating memory cells is now well established (Netea et al., 2016; Nikzad et al., 2019). Since NK cells are efficient eliminators of HCMV-infected cells, it is not surprising that HCMV has devised multiple strategies to evade recognition by these cells (Babić et al., 2011; Goodier et al., 2018; Zingoni et al., 2018). Likewise, APCs from the myeloid and epithelial compartments [i.e., monocytes, macrophages, and dendritic cells (DCs)], are well-known targets of HCMV, serving as vehicles upon infection to facilitate viral dissemination (Jackson and Sparer, 2018). In particular, HCMV is able to interfere with MHC class I (MHC-I) and II (MHC-II) antigen presentation, thereby subverting the immunological functions of APCs.

This review provides an in-depth description of the complex interplay between the host innate immune responses and HCMV, highlighting multiple viral feedback mechanisms that modulate and counteract the various arms of innate immunity.

THE IFN SYSTEM AND HCMV: A STORMY RELATIONSHIP

Upon HCMV sensing, intracellular pattern recognition receptors (PRRs) trigger downstream signaling events leading to the production of type I IFN and release of inflammatory cytokines. Type I IFNs (IFN-I) are a group of cytokines comprising IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-δ, IFN-ζ, and IFN-τ (Mesev et al., 2019).

IFN-I signaling pathways have long been considered key limiting factors of HCMV infection and replication. Despite their complexity, these defense mechanisms occur early after pathogen entry into the host and, in most cases, they can eradicate the pathogen before it can overwhelm the host immune defenses (Goodwin et al., 2018).

Cellular sensors capable of detecting HCMV include toll-like receptor 2 (TLR2) and CD14 receptors, both able to interact

with HCMV envelope glycoproteins (Compton et al., 2003), most of DNA sensors and the newly described group of PRRs, able to stimulate transcription of IFN-I *via* the key adaptor protein stimulator of interferon genes (STING). In particular, the DNA sensor cyclic guanosine monophosphate (GMP)–adenosine monophosphate (AMP) synthase (cGAS)/STING axis is crucial for activating the IFN-I signaling (Diner et al., 2016; Paijo et al., 2016; Jønsson et al., 2017; Biolatti et al., 2018b). On the other hand, HCMV has evolved a wide range of proteins with which to manipulate and counteract the host IFN response (Biolatti et al., 2018c; Goodwin et al., 2018; Marques et al., 2018; Stempel et al., 2019). This complex and intertwined relationship between HCMV and IFN has been addressed by a number of studies discussed below and schematically represented in **Figure 1**.

The HCMV tegument protein pp65 –also identified as pUL83, encoded by *UL83* – best exemplifies the multifaceted interplay between viral and host proteins (Biolatti et al., 2018a). Specifically, pp65 has been shown to modulate nuclear factor-κB (NF-κB) and interferon regulatory factors 3 (IRF3) activities, which cooperate to induce transcription of several cytokines such as IFN-β, which then counteracts HCMV infection (Iwanaszko and Kimmel, 2015).

The recent finding that IκB kinases, the main regulators of NF-κB pathway, exerts antiviral activity (Goodwin and Munger, 2019) adds a level of complexity to this scenario. In this regard, pp65 is able to inhibit NF-κB but not IRF3 nuclear translocation (Browne and Shenk, 2003). This is in disagreement with findings by Abate et al. (2004) showing that pp65 reduces IRF3 phosphorylation preventing its nuclear translocation.

Recent results obtained by our group have demonstrated that the pyrin association domain (PAD) of pp65 binds cGAS, thereby inhibiting its enzymatic activity upon HCMV infection. This phenomenon leads to impairment of the cGAS/STING axis and downregulation of IFN-β production (Biolatti et al., 2018b). In good agreement with these findings, the HCMV tegument protein pUL31 (encoded by *UL31*), similar to pp65, can interact with nuclear and cytoplasmic cGAS in HCMV-infected HFFs and HEK293T cells. Results from Huang et al. (2018) have shown how pUL31 can interact directly with cGAS in HEK293T cells, which is followed by disassociation of DNA from cGAS leading to decreased cGAMP production and consequent downregulation of IFN-I gene expression.

The HCMV tegument protein pp71 (i.e., pUL82, encoded by *UL82*) also contributes to evade the IFN response. According to Fu et al. (2017), pp71 interacts with the inactive rhomboid protein 2 (iRhom2) and STING to disrupt STING trafficking. Particularly, pp71 prevents STING translocation from the ER to the perinuclear microsomes, an essential step of STING-mediated signaling.

The HCMV glycoprotein US9, encoded by *US9*, inhibits IFN-I by targeting mitochondrial antiviral-signaling protein (MAVS) and STING pathways (Choi et al., 2018). In this regard, Choi et al. (2018) have proposed that US9 inhibits IRF3 nuclear accumulation by preventing STING dimerization. Moreover, the overexpression of US9 disrupts the mitochondrial membrane integrity and its membrane potential.

Moreover, the HCMV immediate early (IE) 86 kDa protein (IE86), negatively affects IFN-β mRNA transcription by preventing NF-κB binding to the IFN-β promoter (Taylor and Bresnahan, 2006). Intriguingly, a recent study by Kim et al. (2017) has shown that IE86 downregulates STING protein, suggesting that IE86 may also target STING for proteasomal degradation. Interestingly, STING levels were restored upon treatment with the peptide aldehyde MG132, which prevents the proteolytic activity of the proteasome complex. However, no interaction between STING and IE86 during HCMV infection could be detected.

Finally, HCMV tegument proteins have also been proposed to affect the modulation of type II IFN (also known as IFN-γ) signaling, which is an aspect not well studied. In this regard, Feng et al. (2018) have reported that the human N-myc interactor (Nmi) protein, which is important for the activation of IFN-γ, specifically interacts with the viral tegument protein UL23, encoded by *UL23*, leading to a decrease in IFN-γ expression, thus facilitating viral immune evasion.

To summarize, HCMV has evolved sophisticated mechanisms to modulate the host IFN response, especially that through IFN-I. This new evidence contributes to our understanding of the molecular mechanisms employed by HCMV to evade the innate immune response (**Table 1**).

RESTRICTION FACTORS VS. HCMV: A NEVER ENDING FIGHT

During the last few years, RFs have emerged as main players of the host antiviral response against HCMV (Paludan et al., 2011). RFs are intrinsic antiviral factors, which are sometimes regarded as integral part of the innate immune response or some other times an autonomous third branch of the immune system (Yan and Chen, 2012). Unlike other classical components of innate immunity, they are constitutively expressed within the host cells and are generally IFN inducible, thus allowing an immediate response against viral infection through specific targeting of viral/cellular components (Bieniasz, 2003; Hotter and Kirchhoff, 2018). Interestingly, during HCMV infection a subset of classical IFN-stimulated genes (ISGs) may be also induced or upregulated independently of IFN (Ashley et al., 2019).

Similar to what observed for the IFN system, HCMV has devised clever strategies to sidestep the antiviral activity of RFs, among which IFN-γ-inducible protein 16 (IFI16), nuclear domain 10 (ND10) and virus inhibitory protein ER-associated IFN-inducible (viperin) are among the best characterized (Biolatti et al., 2018c). This list has been in the last few years expanded to include apolipoprotein B editing catalytic subunit-like 3 (APOBEC3), survival time-associated PHD protein in ovarian cancer 1 (SPOC1), Galectin-9 (Gal-9) and human myxovirus resistance 2 (MX2) gene product MxB (**Figure 2**).

Unexpectedly, BST2/tetherin, considered to be the pioneer among RFs due to its long established antiviral activity against human immunodeficiency virus (HIV), does not display restriction activity against HCMV, but it rather enhances the susceptibility of hematopoietic cells to HCMV infection, thereby

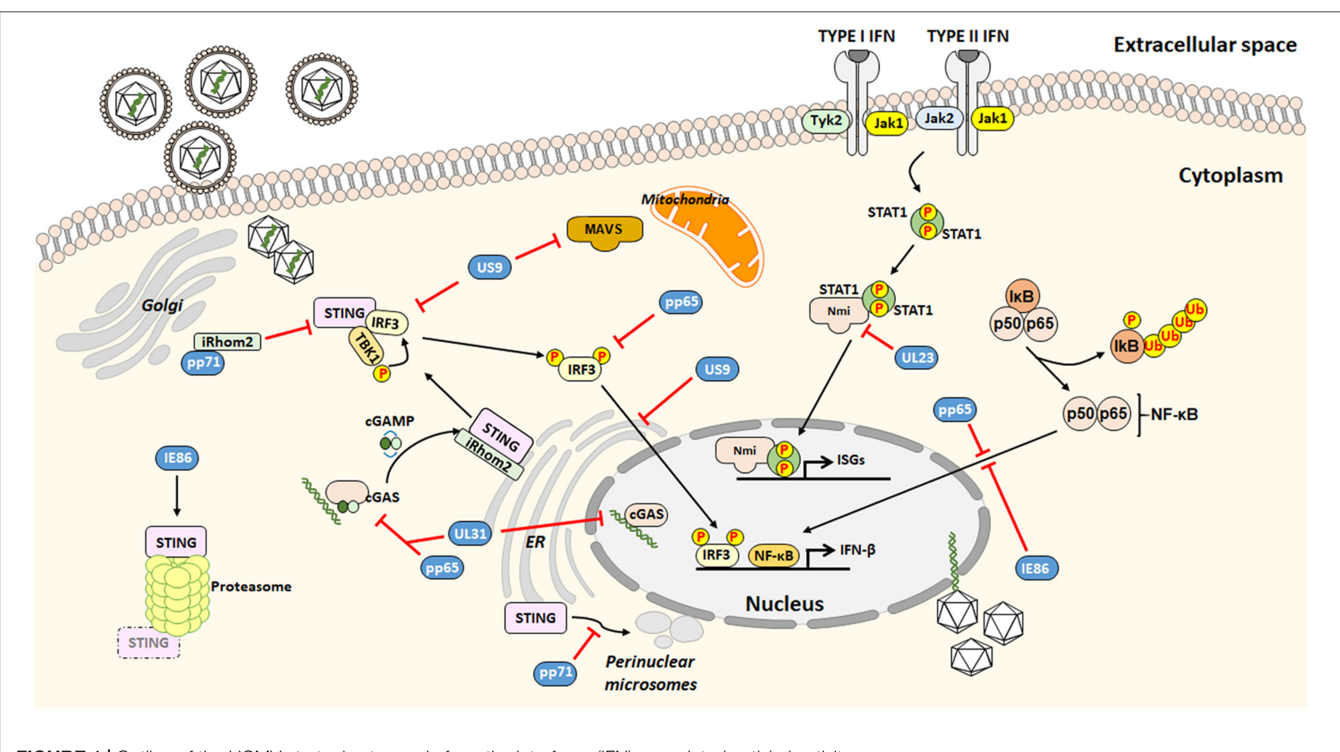


FIGURE 1 | Outline of the HCMV strategies to evade from the interferon (IFN)-associated antiviral activity.

TABLE 1 | Summary of studies describing HCMV evasion strategies from IFN antiviral activity.

Viral protein (viral gene)	Host target	Suggested mechanism	Type of IFN	References
pp65 (UL83)	NF-κB	Reduced nuclear relocalization	IFN-β	Browne and Shenk, 2003
	IRF3	Reduced phosphorylation and relocalization	IFN-β	Abate et al., 2004
	cGAS	Reduced enzymatic activity	IFN-β	Biolatti et al., 2018a
pUL31 (UL31)	cGAS	Dissociation of cGAS from DNA	IFN-β	Huang et al., 2018
pp71 (UL82)	iRhom	Distruption of translocation complex	IFN-β	Fu et al., 2017
	STING	Distruption of translocation complex	IFN-β	
US9 (US9)	MAVS	Attenuation of MAVS signaling	IFN-β	Choi et al., 2018
	STING/TBK1	Prevention of STING oligomerization	IFN-β	
	IRF3	Dysfunctional nuclear relocalization	IFN-β	
IE86 (UL122)	NF-κB	Preventing interaction with IFN-β promoter	IFN-β	Kim et al., 2017
	STING	Proteasome degradation	IFN-β	Taylor and Bresnahan, 2006
UL23 (UL23)	Nmi	Disruption of Nmi/STAT1 interaction	IFN-γ	Feng et al., 2018

favoring viral hematogenous spread (Viswanathan et al., 2011). Similarly, IFN-inducible transmembrane proteins (IFITMs) 1, 2, and 3, capable of blocking the entry of a broad variety of RNA viruses, fail to inhibit the entry of DNA viruses, such as HCMV, HPV16 and human adenovirus type 5, pointing to an evolutionarily preserved mechanism shared by some DNA viruses to circumvent the antiviral function of IFITMs (Warren et al., 2014). This is however a controversial point, as a more recent study has shown that HCMV, instead of taking part in the entry process, exploits IFITMs at later time points of its viral cycle to facilitate the formation of the virion assembly compartment (vAC), which enhances virion assembly (Xie et al., 2015). Finally, a very recent work elegantly described the ability of HCMV to actively stimulate the cellular RNA-binding protein Roquin in inhibiting the innate immune response through the suppression of IRF1 antiviral activity (Song et al., 2019).

IFI16

In the past decade, our group and others have extensively investigated the antiviral activity of IFI16 against HCMV. In particular, we have shown that IFI16 inhibits HCMV replication at early-late phases through blockade of Sp1 binding to the HCMV DNA polymerase promoter (UL54) (Gariano et al., 2012). At late stages of infection, we also found that HCMV is able to promote IFI16 nuclear delocalization through UL97-mediated IFI16 phosphorylation. Phospho-IFI16 is then redirected from the nucleus to the vAC where it is incorporated into newly formed viral particles (Dell'Oste et al., 2014).

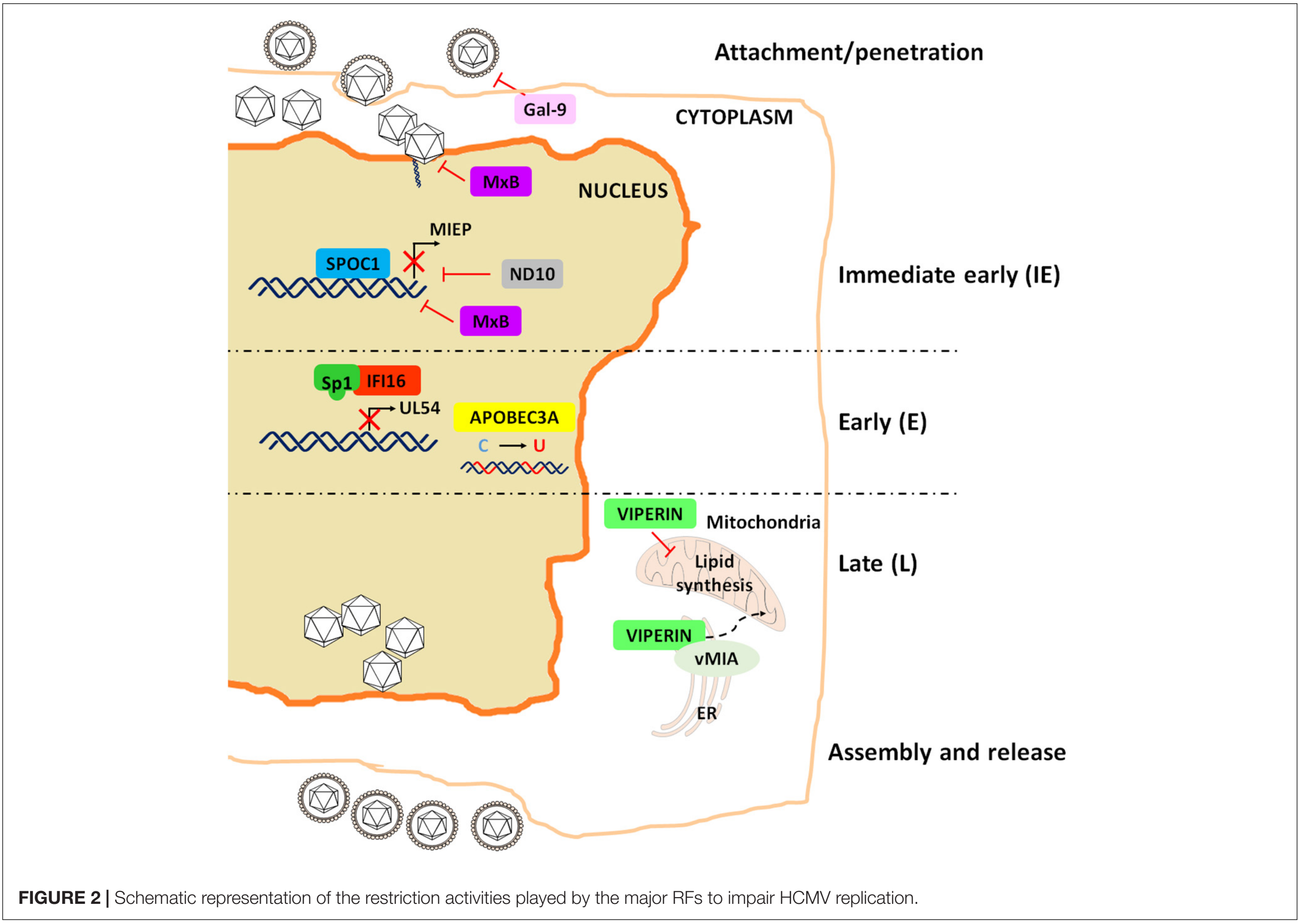


FIGURE 2 | Schematic representation of the restriction activities played by the major RFs to impair HCMV replication.

This unexpected behavior raised the important question of why HCMV chooses to incorporate an RF (i.e., IFI16) into its virions. A partial answer to this riddle came from experiments on pp65 showing that at early stages of HCMV infection this tegument protein can interact with IFI16 at the major immediate-early promoter/enhancer (MIEP), promoting viral gene transcription. Thus, entrapping cytoplasmic IFI16 into virions might after all confer a fitness advantage to the virus (Cristea et al., 2010). However, more recent findings have shown that pp65 can also protect IFI16 from degradation, thereby favoring the inhibitory effect of this latter on the promoter region of *UL54* (Biolatti et al., 2016). Interestingly, it has been recently demonstrated that IFI16 is rapidly targeted during the establishment of viral latency in a US28-dependent manner, but only in undifferentiated myeloid cells, a natural site of latent carriage (Elder et al., 2019). These authors have indeed proposed that the consequent downregulation of IFI16 is beneficial to the establishment of latency, since IFI16 overexpression drives MIEP activity and IE gene expression via NF-κB.

In addition to its antiviral activity, IFI16 is also able to induce IFN-β expression through cGAS interaction (Diner et al., 2016). cGAS activity plays a major role in the STING/tank-binding kinase (TBK-1)/IRF3 pathway, activated by herpes simplex virus type 1 (HSV-1) and HCMV infection (Diner et al., 2016; Biolatti et al., 2018c). Therefore, it does not come as a surprise that also in this case HCMV has been able to develop a strategy to counteract cGAS activity. Indeed, HCMV UL31 has been recently identified as a cGAS inhibitor, acting through direct protein-protein interaction followed by DNA dissociation from cGAS and reduced cGAMP production (Huang et al., 2018).

ND10 Complex

One of the best characterized HCMV RFs is certainly the ND10 complex, formed by the proteins PML, hDaxx, and Sp100 (Zhang and van Drunen Littel-van den Hurk, 2017). In addition to these components, other molecules, such as the nuclear matrix protein microrchidia family CW-type zinc-finger 3 (MORC3/NXP-2), have been shown to associate with the ND10 complex and exert antiviral activity through an unknown mechanism (Sloan et al., 2016).

During HCMV infection, the viral genome is accumulated at the periphery or within the central core of ND10 bodies, and all the ND10 components are recruited at the site of viral replication to exert their antiviral activity (Tavalai et al., 2008; Adler et al., 2011; Cosme et al., 2011; Glass and Everett, 2013). This is achieved by forming a transcriptionally inactive chromatin complex binding the MIEP, which then silences IE gene expression (Preston and Nicholl, 2006; Woodhall et al., 2006;

Lukashchuk et al., 2008; Shin et al., 2012). Moreover, PML is an E3 ligase mediating IE1 SUMOylation, thereby blocking the antagonistic effect of IE1 on STAT-mediated IFN response (Reuter et al., 2017).

Although PML, hDaxx, and Sp100 act as RFs during HCMV lytic replication, they do not seem to affect HCMV latency, as demonstrated by silencing experiments in non-differentiated THP-1 monocytes (Wagenknecht et al., 2015). Meanwhile, other have shown that hDaxx can act as an RF in several latency cellular models, such as NT2 and THP-1 cells, myeloblastic cell lines and primary human CD34^{+} cells (Saffert and Kalejta, 2006).

Also in this instance, HCMV has developed fine-tuned strategies to subvert the gatekeeping functions of ND10. Perhaps the most surprising solution adopted by HCMV relies on IE1, probably because this viral protein is also the main target of the ND10 complex. Specifically, IE1 can block ND10 SUMOylation (Xu et al., 2001; Lee et al., 2004; Schilling et al., 2017), thereby preventing ND10 oligomerization and activation (Korioth et al., 1996; Ahn and Hayward, 1997; Wilkinson et al., 1998). Moreover, the viral latency-associated gene product (LUNA), encoding a deSUMOylase activity, promotes the disruption of cellular ND10 bodies during latency (Poole et al., 2018).

Other strings to the bow of HCMV are its tegument proteins. Indeed, HCMV pp71 prevents hDaxx-mediated repression of MIEP by binding this protein and stimulating its proteasome degradation, leading to disruption of the ND10-MIEP complex (Hofmann et al., 2002; Cantrell and Bresnahan, 2005). In addition, two other tegument proteins, UL35 and UL35a, have been found to cooperate in regulating pp71activity. In particular, UL35 interacts with pp71, and this interaction has two different effects: at early steps of viral replication, this complex activates IE gene transcription (Schierling et al., 2004), whereas at later stages UL35 independently remodels ND10 and co-localizes with the remodeled structures, thus facilitating pp71-mediated hDaxx disruption. Intriguingly, this activity appears to be negatively regulated by UL35a, which prevents UL35 from shaping ND10 and delivers pp71 to the cytoplasm (Salsman et al., 2011).

Viperin

Another early identified HCMV RF is the IFN-inducible iron-sulfur (4Fe-4S) cluster-binding protein viperin, whose main antiviral activity is exerted during late phases of HCMV life cycle (Chin and Cresswell, 2001). A curious aspect of this interplay is that HCMV is not just able to inhibit viperin RF activity but it has learned how to take advantage of it in different ways. Firstly, HCMV promotes viperin translocation from the ER to the mitochondria by encoding the viral mitochondria-localized inhibitor of apoptosis (vMIA) protein. Once in the mitochondria, viperin can inhibit viral replication by modulating the host metabolism through three distinct mechanisms: (1) inhibition of fatty acid β-oxidation; (2) downregulation of ATP levels; and (3) rearrangement of the actin cytoskeleton (Seo et al., 2011). To this end, viperin transcriptionally activates several mediators of fatty acid metabolism, such as AMP-activated protein kinase (AMPK) and GLUT4 (Seo and Cresswell, 2013). This processes leads to enhanced lipid production in HCMV-infected cells, which in turn favors viral envelope formation and virion release.

APOBEC3

Together with tetherin, cytidine deaminases belonging to the APOBEC3 family are considered fundamental antiviral proteins, known for their antiviral activity against HIV-1 (Blanco-Melo et al., 2012). Over the years, their antiviral activity has also been shown to affect DNA viruses, including HCMV (Harris and Dudley, 2015). Specifically, the APOBEC3 family member APOBEC3A (A3A) is upregulated in the maternal decidua upon HCMV infection or IFN-β administration and displays a strong inhibitory effect against HCMV replication (Weisblum et al., 2017). Furthermore, A3A cytidine deamination activity is responsible for hypermutations in the viral genome of HCMV-infected epithelial cells, thereby impairing HCMV replication through a poorly defined mechanism, presumably involving IFN-β (Weisblum et al., 2017).

The observation that A3A is not the only APOBEC3 isoform induced by HCMV comes from one of our recent studies showing that A3G is also strongly upregulated in HCMV-infected HFFs, an induction apparently mediated by IFN-β (Pautasso et al., 2018). However, the fact that the HCMV genome almost totally lacks A3G motifs (i.e., CCC) rules out the possibility that this protein is a *bona fide* HCMV RF, raising the hypothesis that host-virus coevolution might have shaped the nucleotide composition of HCMV DNA to generate viruses able to dodge A3G-mediated immune surveillance.

SPOC1

SPOC1, also known as PHF13 (PHD finger 13), was characterized for the first time in patients with epithelial ovarian cancer (Mohrmann et al., 2005). Many cellular functions of this protein can be attributed to its ability to bind and modulate chromatin by cooperating with several heterochromatin proteins. By doing so, SPOC1 differentially regulates subsets of genes mainly involved in DNA binding and chromatin organization, cell cycle and differentiation (Kinkley et al., 2009; Bördlein et al., 2011; Chung et al., 2016). SPOC1 is also a DNA repair factor as it accumulates at DNA double-strand breaks and regulates the DNA damage response (Mund et al., 2012). A restriction activity of SPOC1 has been observed against human adenovirus type 5 (HAdV5) (Schreiner et al., 2013) and HIV-1 (Hofmann et al., 2017). In these specific contexts, SPOC1 inhibits viral replication, but it is also degraded by viral proteins as a negative feedback mechanism. Furthermore, SPOC1 inhibits early steps of HCMV replication by specifically binding MIEP and driving the recruitment of heterochromatin-building factors, in line with its chromatin remodeling activity. Intriguingly, HCMV but not HIV-1 and AdV5 infection promotes and early transient upregulation of SPOC1 through an IE1-mediated mechanisms independent of protein stabilization. At later steps of infection, SPOC1 levels start to decline upon phosphorylation by the serine-threonine kinase glycogen synthase kinase 3β (GSK-3β) (Hofmann et al., 2017). However, contrary to HIV-1 infection, where Vpr has already been identified as the viral protein involved in SPOC1 degradation (Reichel et al., 2018), the mechanism of HCMV-mediated downregulation of SPOC1 still remains obscure.

Gal-9

Among the most recently identified HCMV-RFs, Gal-9 is of particular interest. It belongs to the widely expressed protein family of galectins, playing an important role in both innate and adaptive immunity (Rabinovich et al., 2007; Rabinovich and Toscano, 2009). The immunomodulatory role of Gal-9 is due to the presence of glycan structures on the surface of both host cells and microorganisms, thus enabling galectins to orchestrate antiviral immunity as well as host-virus interactions. For example, Gal-1 and Gal-9 have shown antiviral activity against Epstein-Barr virus (EBV), murine CMV infection (MCMV), Nipah virus (NIV), enterovirus, HIV-1, influenza virus, and dengue virus in a number of *in vivo* and *in vitro* models of infection (reviewed in Merani et al., 2015).

Even though galectins can either enhance or inhibit viral infection, a restriction activity of Gal-9 during HCMV infection has been recently observed (Machala et al., 2019). In experiments where Gal-9 was added at different time points after HCMV infection it functioned as an antiviral lectin binding the virions and blocking entry of HCMV into the host cell without influencing post-entry events (Machala et al., 2019). On the other hand, the same authors observed increased concentrations of soluble Gal-9 in the plasma of hematopoietic stem cell transplantation (HSCT) recipients during HCMV reactivation, raising the possibility that Gal-9 may also exert a restriction activity *in vivo* (Machala et al., 2019).

MxB

The Mx GTPases MxA and MxB are best known as RFs of several RNA viruses, including influenza A virus, vesicular stomatitis virus (VSV), measles virus (MeV) (Haller and Kochs, 2011), and HIV-1 (reviewed in Staeheli and Haller, 2018). The antiviral activity of Mx against herpesviruses is somewhat more controversial. Indeed, while it has recently been demonstrated a pan-herpesvirus restriction activity for MxB against IE viral gene expression, the precise mechanisms it relies on has not yet been fully clarified (Schilling et al., 2018). The most consistent hypothesis is that of a direct action of MxB during the uncoating process aimed at targeting viral capsids or components of the nuclear pore complexes, similarly to what happens during HSV-1 infection (Crameri et al., 2018).

ANTIGEN PRESENTING CELLS: A TWO-EDGED SWORD

APCs are often defined as sentinels of the body, essential for initiating the immune response against pathogens. They, however, play an enigmatic role during HCMV infection. On the one hand, many APCs, including monocytes, macrophages and DCs, are critical to trigger specific T-cell responses. On the other hand, they are permissive to HCMV infection, serving as vehicles for viral spread during the first steps of infection, and then becoming cozy and protective niches for virus replication and persistence at later stages. Conversely, components of the lymphoid lineage, such as NK cells and plasmacytoid DCs (pDCs) are not just resistant to HCMV infection but they are also activated early upon infection by viral components, triggering an antiviral response. Despite the presence of these defense mechanisms, HCMV has put in place multiple strategies to evade APC-mediated immune control so as to establish latency and persistence within the host (Sinclair and Reeves, 2014).

Dendritic Cells (DCs)

DCs are specialized APCs mediating immune response induction and maintenance. The major subsets in humans include classical DCs (cDCs), which comprise Langerhans cells (LCs) and pDCs, the main producers of IFN-I, and monocyte-related DCs (mDCs) (Collin et al., 2013). The role of DCs during HCMV infection remains somewhat controversial because, despite being critical components for the establishment of an antiviral NK and T-cell response, they are also targeted by HCMV for immune escape.

HCMV interacts with DCs in a pleiotropic manner. It is in fact well established that HCMV strains with an intact UL128-131A locus can infect DCs *in vitro* (Jahn et al., 1999; Riegler et al., 2000). In addition, circulating mDCs isolated from healthy seropositive donors can also support HCMV infection (Reeves and Sinclair, 2013), a process probably favored by the expression of the viral chemokine receptor-like protein US28, which drives DC recirculation (Farrell et al., 2018). In contrast, by using co-culture approaches, it has been shown that mDCs or monocyte-derived macrophages can restrict HCMV with interferon-unrelated mechanisms (Kasmapour et al., 2017; Becker et al., 2018).

For pDCs, the scenario is even more complex. Different subpopulations of pDCs obtained either from tonsils (tpDCs) or blood (bpDCs) react to HCMV-infection in opposite ways (Schneider et al., 2008). For instance, tpDCs are fully permissive for HCMV replication despite the fact that their IFN-α production and expression of costimulatory and adhesion molecules are ultimately affected by HCMV. In contrast, bpDCs appear to be resistant to HCMV infection (Schneider et al., 2008).

HCMV can latently infect DC precursors and then undergo reactivation by taking advantage of chromatin remodeling during differentiation of DC progenitors into mature DCs (Reeves et al., 2005). Conversely, in undifferentiated myeloid precursors, viral lytic genes are inhibited as a consequence of histone modifications of the MIEP, leading to a repressive chromatin structure eventually preventing IE transcriptional activity (Sinclair, 2010). Furthermore, proinflammatory factors, such as IL-6 and the ERK/MAPK pathway have been linked to the reactivation of latent HCMV in DCs and other permissive cells (Reeves and Compton, 2011).

The interplay between HCMV and DCs interaction can have different outcomes in terms of immune response. For instance, HCMV infection of mDCs *in vitro* triggers IFN and IL-12 release in a cGAS-dependent manner (Renneson et al., 2009; Paijo et al., 2016). Subsequently, other immune mediators are recruited to the infection site to amplify the immune reaction. HCMV infection in mDCs can also modulate TLR3 signaling, but this effect is more evident at later times post-infection (Mezger et al., 2009).

Given the central role of DCs in virus clearance, it is not surprising that HCMV has put in place multiple strategies

to inhibit such process. For instance, HCMV can interfere with MHC-I and -II antigen processing and presentation to avoid detection by $CD8^+$ and $CD4^+$ T cells. This process appears to be mediated by the HCMV-encoded protein US2, capable of degrading both MHC-I and MHC-II proteins through the proteasome (Loureiro and Ploegh, 2006). Likewise, other HCMV proteins such as pp65, pp71, and US2-11 have been implicated in HCMV evasion from T-cell recognition by triggering accumulation and degradation of HLA-DR α-chain in perinuclear vacuoles (Odeberg et al., 2003).

Among HCMV genes hindering APC function, a crucial role is played by the viral interleukin-10 homolog (cmvIL-10), expressed during lytic infection and capable of binding the IL-10 receptor of host cells. Specifically, cmvIL-10 upregulates the HCMV putative receptor DC-SIGN, thus enhancing viral infectivity (Raftery et al., 2004), as well as the expression of hIL-10 by primary blood-derived monocytes, thus modulating existing cellular pathways and the viral immunomodulatory impact during infection (Avdic et al., 2016). In addition, it inhibits a number of DCs functions, including TLR-induced IFN-α/β production in nearby pDCs and CD1-mediated antigen presentation (Raftery et al., 2008; Avdic et al., 2014). This effect is also shared by other viruses, which either upregulate hIL-10 (e.g., HIV and hepatitis C virus) (Reiser et al., 1997; Brockman et al., 2009) or express homologs of this cytokine (e.g., EBV and some cytomegaloviruses) (Slobedman et al., 2009), highlighting the importance of IL-10 signaling in viral escape mechanisms.

An important step of the immune response is the ability of DCs to drift from the infection site to the lymph nodes, a process driven by the chemokines CCL19 and CCL21. Consequently, HCMV has developed strategies to impede DC trafficking in response to lymphoid stimuli and induction of T-cell proliferation (Beck et al., 2003; Moutaftsi et al., 2004). For example, it can prevent CCR5 chemokine receptor from switching to CCR7 in infected mDCs, thus inhibiting CCL19- and CCL21-induced migration of mature mDCs (Moutaftsi et al., 2004). Conversely, in immature mDCs, HCMV does not modulate CCR7, but it affects chemotaxis by internalizing CCR1 and CCR5 (Varani et al., 2005). In this context, UL18, the viral homolog of MHC-I, appears to play a controversial role. Indeed, UL18 has been reported to inhibit CD40L-mediated T-cell proliferation through DC maturation impairment (Wagner et al., 2008), meanwhile stimulating the expression of CD83 on mature mDCs. Moreover, at later times, HCMV downregulates surface but not intracellular CD83 (Wagner et al., 2008). Others have reported that soluble CD83, in turn, inhibits T-cell proliferation (Sénéchal et al., 2004), and that UL18 is also able to reduce RANTES-driven chemotaxis of mDCs (Wagner et al., 2008; **Figure 3**).

Depending on their stage of maturation, $CD34^+$ progenitor cell-derived LCs can be susceptible to HCMV infection. Indeed, immature LCs are poorly supportive of viral replication, whereas LC-derived mature DCs are highly responsive to infection due to HCMV-mediated subversion of the T-cell response through downregulation of several activation markers, such as MHC-I and -II, CD1a, CD80, CD83, CD86, and CD54 (Hertel et al., 2003). This also leads to a substantial loss of dendrites and to impaired dendritic cell migration in response to lymphoid chemokines (Lee et al., 2004; **Figure 3**).

Monocytes and Macrophages

Additional reservoirs for HCMV are represented by monocytes and macrophages. In particular, monocytes have been long involved in HCMV dissemination across the human body and are generally regarded as the main source of latent HCMV in the peripheral blood of seropositive people (Smith et al., 2004). Even though they do not support productive HCMV replication (Sinzger et al., 2008), once fully differentiated into macrophages, they become permissive for viral replication. During this process, a major role for virus reactivation and growth seems to be played by IFN-γ and tumor necrosis factor (TNF)-α, produced by allostimulated T cells (Söderberg-Nauclér et al., 1997). Moreover, monocytes are known to release infectious HCMV directed toward uninfected cells *in vitro* through a not fully defined mechanism (Waldman et al., 1995).

Like DCs, monocyte-derived macrophages play a crucial role in counteracting HCMV spread *in vitro*. In this context, the role of IFN is controversial. Indeed, IFN-I plays an inhibitory role on HCMV replication when macrophages are stimulated by cell-free HCMV. In contrast, upon co-culture of infected cells and macrophages, the antiviral effect appeared to be independent of IFN-γ, TNF-α, and IFN-I (Becker et al., 2018).

Overall, it seems that HCMV has learned how to escape from monocyte antiviral activity and use these cells as "Trojan horses" to achieve viral spread. For instance, infected monocytes display impaired migration and reduced ability to recruit leukocytes and inflammatory mediators, allowing additional "contact time" to transfer HCMV from infected monocytes to uninfected cells (Frascaroli et al., 2006). Furthermore, the observation that purified pUL128 – i.e., a CC chemokine homolog, part of the HCMV pentamer complex (PC) – triggers monocyte migration *in vitro* through a poorly characterized mechanism suggests that HCMV might be able to attract monocytes to the infection site and favor viral dissemination by secreting specific chemokines (Zheng et al., 2012). In addition, pUS2-US11-mediated MHC downregulation in DCs is only partially functional in macrophages, which therefore retain their ability to activate $CD4^+$ and $CD8^+$ T cells (Frascaroli et al., 2018). Lastly, HCMV inhibits the differentiation of both macrophages and DCs from monocytic precursors after stimulation with IL-4 and GM-CSF, impairing immunological functions (Gredmark and Söderberg-Nauclér, 2003). In this context, the main inhibitors of macrophage differentiation are the cell-surface aminopeptidase N/CD13 and HCMV glycoprotein B (gB) (Gredmark et al., 2004; **Figure 3**).

As for DCs, cmvIL-10 can also impair cytokine production of these cells through inhibition of phosphatidylinositol 3-kinase/Akt signaling (Spencer, 2007), with concurrent downmodulation of integrin-like receptor surface expression [i.e., CD11b/CD18 (CR3) and CD11c/CD18 (CR4)], a process that strongly impairs DC phagocytic activity (Gafa et al., 2005). Finally, downregulation of CCR1 and CCR5 is associated with slower cell migration, reorganization of the cytoskeleton and secretion of soluble inhibitors (Frascaroli et al., 2009; **Figure 3**).

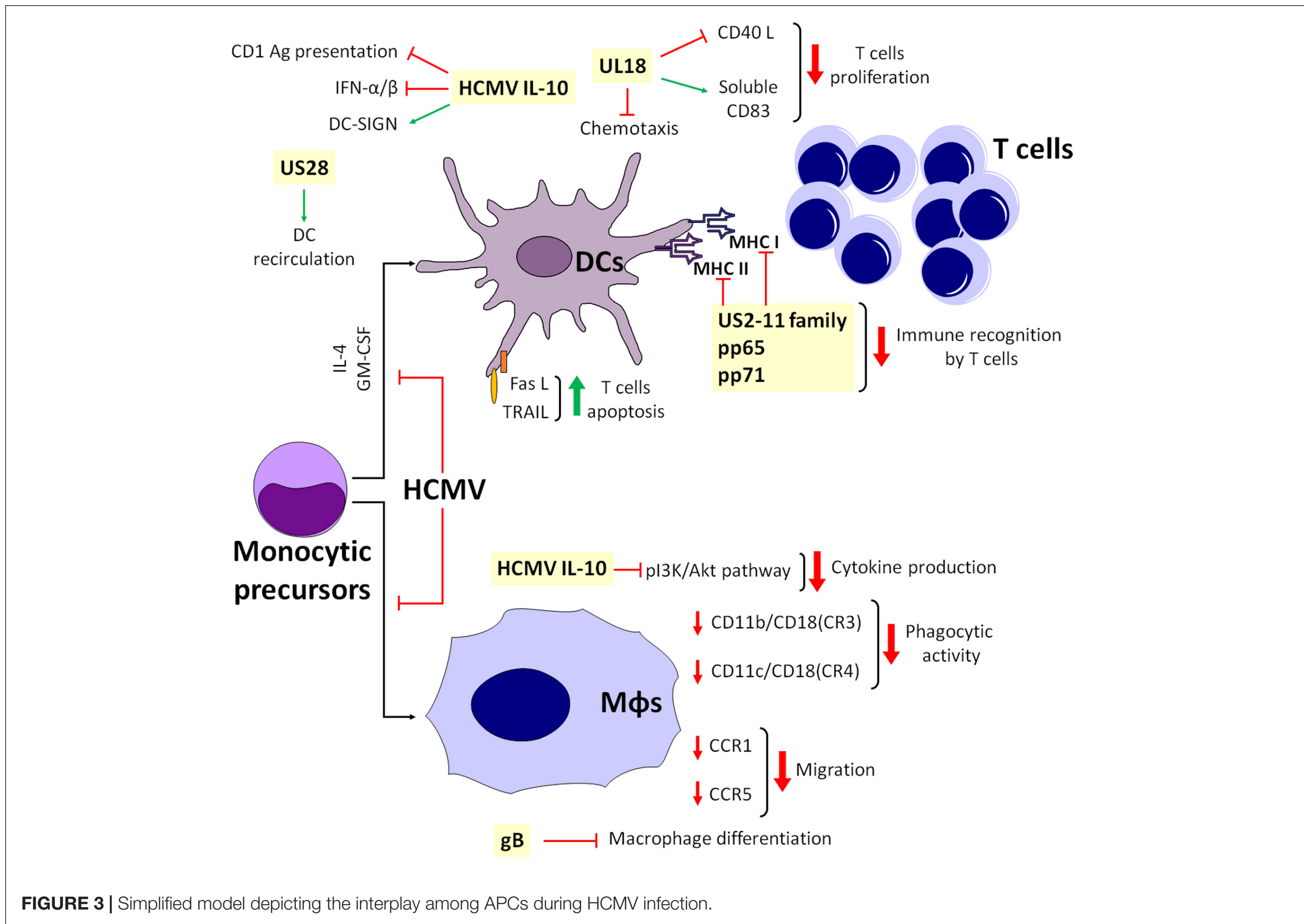


FIGURE 3 | Simplified model depicting the interplay among APCs during HCMV infection.

NK CELLS AND HCMV: A BALANCE OF OPPOSING FORCES

NK cells play crucial role in eliminating HCMV-infected cells through cytotoxicity and secretion of several cytokines and chemokines able to directly impair viral replication (e.g., IFN-g and TNF-a) or to recruit and/or activate other cells of the immune system. However, if on one side there are examples demonstrating the importance of NK cells in controlling HCMV infection, on the other side there is a long list of viral proteins capable of protecting HCMV from NK cell recognition and killing (Brown and Scalzo, 2008; Schmiedel and Mandelboim, 2017; Patel et al., 2018).

The former case is best exemplified by human NK cell primary immunodeficiencies (NKD), which inevitably results in high susceptibility to herpesvirus infections [i.e., HCMV, HSV, EBV, and varicella zoster virus (VZV)] (Biron et al., 1989). In this regard, more than 60% of NKD patients are infected by one of these viruses (Orange, 2013), also in the context of intact CTL functions (Quinnan et al., 1982). The severity of this condition is demonstrated by the fact that nearly half of patients with NKD tend to die prematurely (Orange, 2013; Mace and Orange, 2019).

The antiviral activity of NK cells against HCMV also appears to be mediated by NK cell receptors, whose expression can be to some extent modulated upon viral entry. In particular, HCMV infection can induce the selective expansion of a population of NK cells expressing the activating receptor CD94/NKG2C, giving rise to the so-called "adaptive-like" or "memory-like" NK cells (Gumá et al., 2004). This aspect of NK and HCMV biology is beyond the scope of this review and has already been extensively described in recent reviews (López-Botet et al., 2014, p. 94; O'Sullivan et al., 2015; Rölle and Brodin, 2016).

What is important to point out in this context is that NKG2C receptor skewing is accompanied by other phenotypic, functional and epigenetic modifications, which lead to the generation of a pool of long-living NK cells with increased effector responses upon restimulation. Importantly, Hammer et al. (2018) have recently shown that the triggering event driving NKG2C$^+$ NK cell expansion is mediated by an HCMV-encoded peptide derived from the viral protein UL40 and by the NKG2C ligand HLA-E. However, it is worth pointing out that the emergence of NK cell memory in response to HCMV can also occur in individuals lacking expression of NKG2C – i.e., carrying the null allele KLRC2 encoding for NKG2C – (Noyola et al., 2012), suggesting that alternative or compensatory mechanisms may be in place. This mode of activation is nonetheless complex, as HLA-E is also recognized by CD94/NKG2A, the inhibitory counterpart of CD94/NKG2C, with identical peptide specificity

(Braud et al., 1998; Lee et al., 1998; Brooks et al., 1999; Cerboni et al., 2000; Ulbrecht et al., 2000; Tomasec et al., 2005). Stabilization of HLA-E by the UL40-derived peptide can thus have opposite effects on NK cells, depending on which receptor is involved. However, it seems that the NKG2C$^+$ NK cell population expanding in HCMV seropositive individuals lacks the inhibitory NKG2A heterodimer (Hammer et al., 2018). In addition, the peptide repertoire encoded by different HCMV UL40 variants may result in an intermediate state, where peptides able to efficiently inhibit NKG2A and simultaneously trigger suboptimal activation of NKG2C$^+$ NK cells are more prevalent (Hammer et al., 2018).

The important role of NK cells in CMV infection comes also from a plethora of studies conducted in mice. In general, the absence of NK cells – due to genetic or neutralizing/depleting antibody manipulations – results in a significantly diminished, and sometimes lethal, control of MCMV (Bukowski et al., 1984; Brown and Scalzo, 2008). Similarly to HCMV, it has been reported a pathogen-specific recognition mechanism for protection, involving the NK cell-activating Ly49H receptor, which specifically recognizes the MCMV protein m157 (Arase et al., 2002).

Another important strategy for immune escape is the ability of HCMV to manipulate the expression of several ligands of the NKG2D receptor, expressed on all NK cells, CD8$^+$ T cells and other T-lymphocyte subsets (e.g., CD4 + T cells, gd, and NKT cells) (Lanier, 2015; Zingoni et al., 2018). There are eight different NKG2D ligands (i.e., MICA, MICB, and ULBP1-6), all belonging to the MHC class I-like family and possessing two or three αdomains, but not able to bind peptides or β2-microglobulin. These molecules are also known as "stress-induced ligands" or "induced self" as they are rarely expressed on the plasma membrane of healthy cells but can be rapidly up-regulated upon different types of stress, including those triggered by viral infection (Cerboni et al., 2014; Lanier, 2015). In the absence of a specific viral countermeasure, up-regulation of NKG2D ligands (NKG2DLs) would likely result in the killing of infected cells, as it has been observed in some experimental conditions (Cerboni et al., 2000; Wang et al., 2002; Pignoloni et al., 2016). However, *in vitro* studies have shown that this is not always the case since HCMV encodes at least seven different molecules – among which a few identified very recently – able to inhibit NKG2DL expression, thus conferring protection to the infected cells. In particular, MICA seems to be the most frequently targeted ligand, with UL142, UL148a, US9, US18, and US20 viral proteins dedicated to block its expression at different levels, sometimes in an allelic-specific manner (Schmiedel and Mandelboim, 2017; Patel et al., 2018; **Figure 4**). Although the reason for such a high number of HCMV proteins targeting just one ligand is currently unknown, their existence may be ascribed to the fact that, among NKG2D ligands, MICA has the highest affinity for its receptor (Steinle et al., 2001) as well as the largest number of variant alleles, with more than 100 identified thus far[1]. Based on these findings, it is tempting to speculate that the antiviral activity of MICA may have selected viruses able to block MICA expression and the ensuing NKG2D-mediated killing, and that this in turn might have promoted MICA polymorphism.

Among NKG2D ligands, we find MICB, a polymorphic gene with more than 40 allelic variants, and 6 ULBP genes boasting a total of 16 allelic variants[2] (Radosavljevic et al., 2002). MICB expression is inhibited by miR-UL112, the only HCMV-encoded miRNA described to date targeting this ligand (Stern-Ginossar et al., 2012), and by the viral protein UL16, which is a sort of promiscuous immunoevasin since it can also inhibit the expression of ULBP1, ULBP2, and ULBP6 (Cosman et al., 2001; Kubin et al., 2001; Dunn et al., 2003; Rölle et al., 2003; Wu et al., 2003; Eagle et al., 2009). ULBP3 is instead targeted by UL142, also blocking MICA expression (Ashiru et al., 2009; Bennett et al., 2010). The ability to simultaneously evade multiple cellular pathways has also been reported for US18 and US20, capable of inhibiting both MICA and the NKp30 ligand B7-H6 (Charpak-Amikam et al., 2017; Fielding et al., 2017).

Other targets of HCMV include CD155/PVR and CD112/Nectin-2, two adhesion molecules belonging to the Ig-like superfamily able to bind the activating receptor CD226/DNAM-1 expressed on cytotoxic lymphocytes (**Figure 4**; Shibuya et al., 1996; Bottino et al., 2003; Tahara-Hanaoka et al., 2004). Similar to NKG2DLs, DNAM-1 ligands (DNAM-1Ls) are often induced by cellular stresses and can trigger cytotoxicity and cytokine release (Shibuya et al., 1996; Bottino et al., 2003; Iguchi-Manaka et al., 2008). For this reason, DNAM-1Ls are also targeted by HCMV, with UL141 downregulating both of them, alone or in combination with US2 through different mechanisms (Tomasec et al., 2005; Prod'homme et al., 2007; Hsu et al., 2015). Of note, UL141 is also able to downregulate the TRAIL receptors R1 and R2, thus preventing TRAIL-dependent NK-cell killing (Nemčovičová et al., 2013; Smith et al., 2013). UL141 is thus a remarkable immunoevasion protein as it targets at least four different molecules regulating NK cell-mediated cytotoxicity.

Adhesion molecules involved in the formation of NK-target cell conjugates are also affected by HCMV. In particular, UL148 down-regulates CD58/LFA-3, the ligand of the CD2 receptor expressed by different leukocyte populations, including NK and CD8$^+$ T cells. The CD2/CD58 axis promotes cell-to-cell adhesion and immunological synapse formation, providing an important co-stimulatory signal on effectors (Siliciano et al., 1985; Selvaraj et al., 1987; Browne et al., 1990) (Leitner et al., 2015). More recently, CD2 has been shown to play a role in costimulation of adaptive NK cells (Rölle et al., 2003; Liu et al., 2016). Furthermore, inhibition of CD58/LFA-3 expression by the viral protein UL148 has revealed that the CD2/CD58 axis is also needed for the recognition of HCMV-infected cells by NK cells and HCMV-specific CTLs (Wang et al., 2018).

In summary, it appears that there is a steadily increasing number of HCMV-encoded proteins evading NK cell recognition and killing. However, to date there is no single viral protein or RNA able to interfere with all the molecules involved in the anti-viral NK cell response.

It is also important to point out that development, proliferation and effector functions of NK cells are tightly

[1] http://www.ebi.ac.uk/imgt/hla/html

[2] https://www.ebi.ac.uk/ipd/imgt/hla/

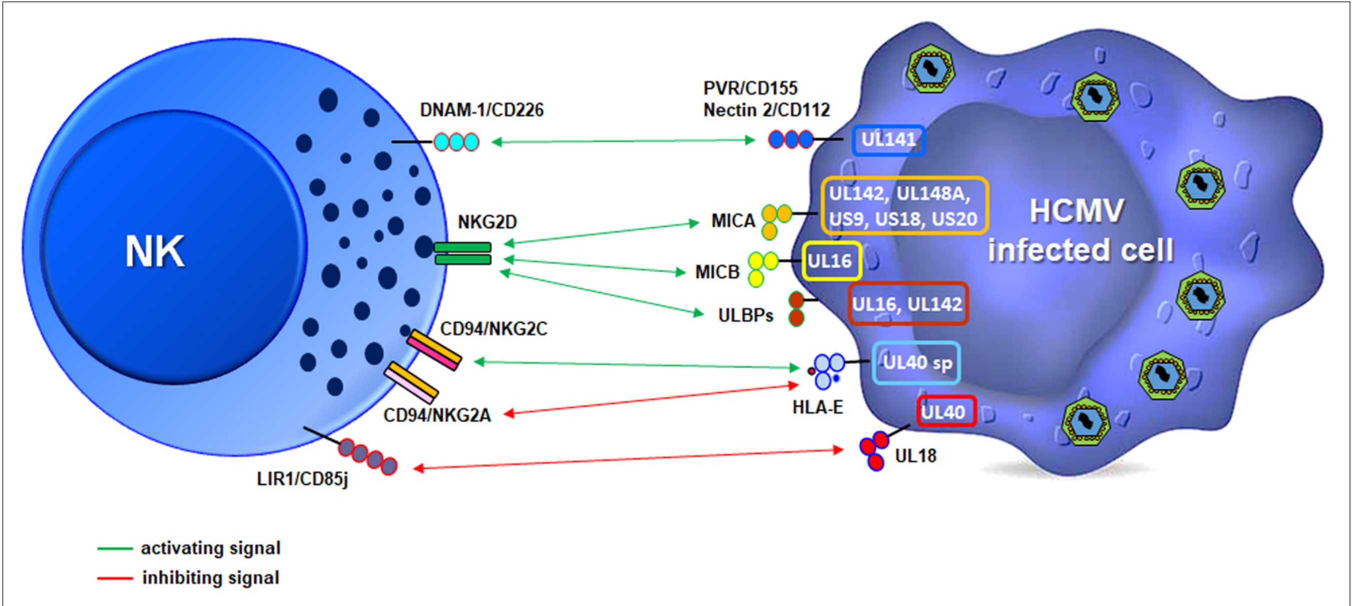


FIGURE 4 | Schematic illustration of the strategies used by HCMV to modulate NK cell receptor ligands. The viral proteins mainly involved are depicted in a representative infected cell (right, color outlines), and activating (green) or inhibitory (red) signals relative to specific receptors on a NK cell (left) are shown.

regulated by both activating and inhibitory receptors, with an outcome that strongly depends on the balance between opposing signals. Inhibition is delivered *via* MHC-I molecules expressed on the surface of target cells. However, HCMV, like many other viruses, negatively affects MHC-I expression in infected cells, as this is a crucial step to avoid cell-mediated killing by viral-specific cytotoxic T cells. In theory, this would render infected cells more susceptible to NK cell recognition due to the absence of inhibitory signals. However, the observation that HCMV-infected cells are resistant to NK lysis *in vitro* seems to suggest otherwise (Cerboni et al., 2000; Wang et al., 2002). What we have in fact described in this section is a plethora of viral molecules evolved by HCMV to escape from NK cell activation, which otherwise would be detrimental for viral fitness.

To complete this picture, HCMV can fully accomplish immunoevasion from NK cells thanks to its own MHC-I surrogate, called UL18. This protein is markedly similar to cellular MHC-I molecules (Beck and Barrell, 1988; Browne et al., 1990) and acts as a viral homolog by binding with high affinity the MHC-I NK cell inhibitory receptor CD85j/LIR1/ILT2, thereby suppressing NK cell functions (Chapman et al., 1999; Cosman et al., 2001; Cerboni et al., 2006; Prod'homme et al., 2007).

In conclusion, HCMV is a driving force in shaping the NK cell receptor repertoire and modes of recognition of infected cells. The virus is not only capable of "hitting the brakes" of NK cells through its own MHC-I surrogate (UL18) or by engaging the CD94/NKG2A inhibitory receptor with UL40, but it can also "block the gas pedal" by inhibiting the expression of several ligands of NK cell activating receptors. The outcome is a million-year-long host-pathogen equilibrium, where neither the host nor the pathogen is at risk of extinction.

HCMV AND APOPTOSIS: "NOT TODAY!"

Apoptosis, or programmed cell death (PCD), is essential for the maintenance of homeostasis and survival of most multi-cellular organisms. Apoptosis occurs predominantly through the following three pathways: (1) extracellular ligand-mediated extrinsic pathway; (2) mitochondria-mediated intrinsic pathway; and (3) ER-mediated pathway. The extrinsic pathway is initiated upon binding of extracellular ligands to death receptors (DRs), leading to the formation of the death-inducing signaling complex (DISC), required for the activation of initiator caspases (i.e., cysteine proteases), caspase-8 and caspase-10. The intrinsic pathway is regulated by B-cell lymphoma 2 (Bcl-2) proteins and is characterized by mitochondrial outer membrane permeabilization (MOMP) (Elmore, 2007). The ER-mediated pathway is instead induced by stress signals, such as excessive unfolded proteins in the ER and triggers the activation of caspases-7, -9, and -12 (Bhat et al., 2017). All these pathways lead to the activation of the executioner caspases-3 and -7 that contribute to the majority of events taking place during apoptosis (Elmore, 2007).

Apoptosis is also one of the main steps of the innate response against viral infections, including HCMV. Also in this case, HCMV has evolved several strategies to subvert host cell apoptotic defenses by targeting key effector molecules in the apoptotic cascade. Upon infection, the slowly replicating HCMV modulates cellular apoptosis pathways in various cell types, such as endothelial cells, fibroblasts and macrophages by encoding numerous death inhibitors to block premature death of host cells, thus favoring its replication (Brune and Andoniou, 2017; Collins-McMillen et al., 2018; **Figure 5**). The following paragraphs will contain a comprehensive review and discussion of some of the

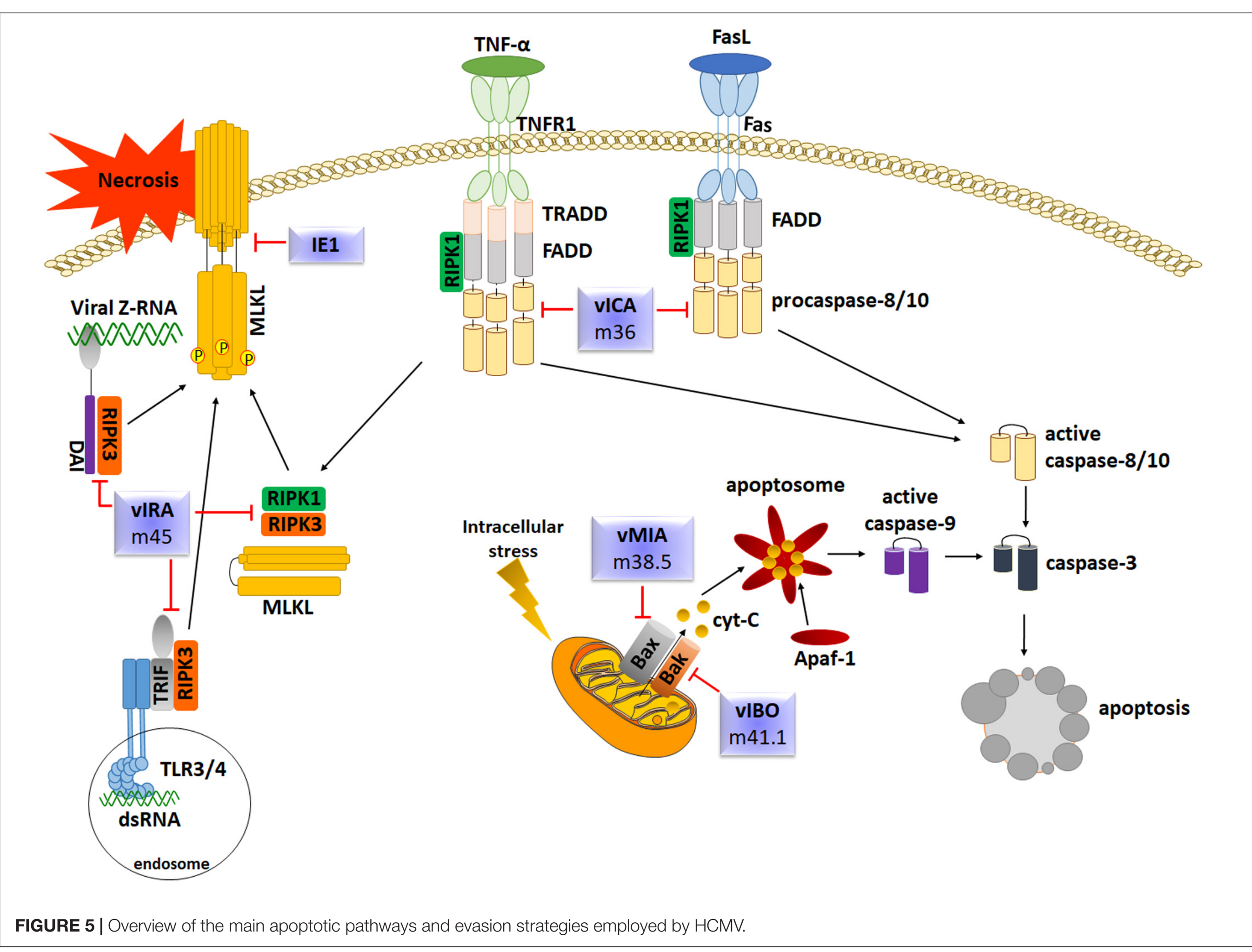


FIGURE 5 | Overview of the main apoptotic pathways and evasion strategies employed by HCMV.

main mechanisms used by HCMV to modulate or prevent the apoptotic pathways of infected host cells.

Inhibition of Extrinsic Apoptosis

Caspase-8 is required for initiation of apoptosis in response to death factors such as Fas-L or TNF-a. Within the Fas-FADD-Caspase-8 complex, also known as DISC, caspase-8 undergoes self-cleavage to convert to the active form. Fully-cleaved caspase-8 is released from DISC to the cytosol to trigger the apoptotic signal to downstream caspase effectors or to cleave the Bcl-2-interacting protein (Bid), which leads to the release of cytochrome c from mitochondria, inducing activation of caspase-9 in a complex with dATP and Apaf-1 (Kruidering and Evan, 2000). To counteract DR- mediated apoptosis and gain a survival advantage, HCMV encodes the viral inhibitor of caspase-8-induced apoptosis vICA/pUL36, which binds the prodomain of procaspase-8, impedes the recruitment of FADD, and prevents the formation of a functional DISC. The fact that homologs of HCMV vICA have been identified in the vast majority of mammalian betaherpesviruses implies that the function of vICA is important and conserved. This is exemplified by M36, the vICA counterpart of MCMV, which also displays an anti-apoptotic activity by interacting with procaspase-8, and that has been shown to be rescued by vICA in order to allow viral replication, confirming the reliability of the murine model (Chaudhry et al., 2017).

Moreover, the replication of UL36-deficient virus can be restored by treatment with the pan-caspase inhibitor z-VAD(OMe)-fluorometyl ketone (fmk) only in immature but not mature macrophages, suggesting that apoptosis impairs the replication of UL36-deficient virus in defined cell types. However, according to McCormick et al. (2010), it seems that cell death pathways activated by HCMV infection are altered as monocytes differentiate to macrophages. Indeed, early during differentiation, UL36-deficient virus-induced apoptosis is dependent on caspases and can be blocked by z-VAD-fmk, while at later stages of differentiation it appears to be caspase-independent.

Inhibition of Intrinsic Apoptosis

Mitochondria play a pivotal role in the intrinsic apoptosis pathway. Initiation and execution of this pathway is regulated by the Bcl-2 effector proteins Bax (Bcl-2-associated X protein) and Bcl-2 antagonist or killer (Bak) that control MOMP. MOMP prompts the release of proapoptotic intermembrane space (IMS)

proteins that promote the formation of the apoptosome – composed by cytochrome c and Apaf-1 – and activation of caspase-9. Once active, caspase-9 can directly cleave the effector caspases 3 and 7 (Estaquier et al., 2012). HCMV prevents MOMP by encoding the viral mitochondria-localized inhibitor of apoptosis (pUL37x1/vMIA). UL37x1, highly conserved among HCMV strains, is located in a complex transcription unit encoding several transcription variants expressed during the IE phase. Two functionally longer splice variants (i.e., gpUL37 and gp37M) share with pUL37x1 an NH2-terminal 162 aa sequence responsible for inhibiting apoptosis, localize partially to mitochondria and have similar, albeit weaker, anti-apoptotic activities (Goldmacher et al., 1999; Colberg-Poley et al., 2000; Reboredo et al., 2004; Kaarbø et al., 2011). pUL37x1 blocks mitochondria-mediated apoptosis by interacting at the level of the mitochondrial outer membrane (MOM) with Bax, thus preventing cytochrome-c release. It still remains to be clarified whether vMIA can inhibit Bak during infection (Sharon-Friling et al., 2006; Sharon-Friling and Shenk, 2014).

Moreover, by using U251 glioma cells a mechanisms of viral apoptosis inhibition and enhancement of cell proliferation has been shown, relying on the activity of the immediate-early protein IE86 on heterogeneous ribonucleoprotein A2/B1 (hnRNP A2/B1) and consequent alternative splicing of Bcl-x (Zhao et al., 2019).

In addition to the aforementioned strategies, HCMV is also involved in preserving the mitochondrial membrane potential and metabolism to prevent cell death. This is achieved thanks to the production of the long non-coding RNA-lncRNA Beta2.7 that enhances cell survival through interaction with gene associated with retinoid/interferon-induced mortality 19 (GRIM19). This interaction causes the stabilization of mitochondrial membrane functions, thereby preserving ATP production and conserving metabolic activity during stress conditions (Poole et al., 2016).

Inhibition of Necroptosis

Necroptosis is an alternative form of programmed cells death that, despite mimicking features of apoptosis, cannot be prevented by caspase inhibitors. Necroptosis can be triggered following activation of DRs as well as after stimulation with LPS, poly(I:C) or CpG DNA, which are ligands of the pattern recognition receptors (PRRs) TLR3, TLR4, and TLR9, respectively. Many downstream signaling pathways cooperate with a complex formed by the receptor interacting protein kinase 1 (RIPK1), RIPK3 and mixed lineage kinase domain-like (MLKL). Necroptosis and apoptosis are strictly interconnected, as confirmed by the observation that the inhibition of caspase-8, the main mediator of the extrinsic apoptotic pathway, promotes the shift from DR-mediated cells death to necroptosis due to activation of RIPK3 and, consequently, MLKL. Phosphorylation of MLKL generates structural changes allowing its insertion into the inner leaflet of the plasma membrane leading to the disruption of cellular membranes (Green, 2019).

Inhibition of Cellular Stress Response

Disturbances of the normal functions of the ER, causing accumulation of unfolded proteins, trigger an evolutionarily conserved cell stress response, known as unfolded protein response (UPR), which, initially aimed to damage compensation, can eventually lead to cell death to avoid viral spread. HCMV prevents this process, in part, *via* UL38, a multifunctional protein well conserved among different CMV species. In particular, viral DNA replication is severely impaired in viruses lacking UL38 (i.e., AD*dl*UL38), a feature associated with enhanced death of infected cells (Terhune et al., 2007). Moreover, pUL38 itself can inhibit cell death induced by thapsigargin, which perturbs calcium homeostasis followed by ER-mediated cell death, or by a mutant adenovirus lacking the antiapoptotic E1B-19K protein. Of note, pUL38 cannot counteract cell death triggered by anti-Fas antibodies (Xuan et al., 2009).

Overall, the aforementioned findings suggest that pUL38 hampers both intrinsic and ER-mediated cell death, but it only slightly affects extrinsic apoptosis. UL38, expressed both at early and late stages of infection, is localized in a complex transcription unit that also retains the unspliced transcripts of UL36 and several variants of UL37, expressed during the IE phase. Probably, pUL36, pUL37x1 and pUL38 act synergically to inhibit cell death at different times during infection. As described above, while pUL36 inhibits caspase-8 activation, pUL37x1 blocks mitochondria-mediated intrinsic apoptosis. Furthermore, UL38 inhibits c-Jun N-terminal kinase (JNK) signaling through interaction with the activating transcription factor 4 (ATF4), which leads to caspase-12 or caspase-2 activation (Xuan et al., 2009).

More recently, Luganini et al. (2018) have shown that HCMV encodes for a viral-Ca^{2+}-permeable channel, pUS21, able to reduce Ca^{2+} content of intracellular stores and to protect cells from apoptosis. Among the US12 gene family members, which includes a set of 10 contiguous tandemly arranged genes (US12-21), pUS21 shows the highest level of identity with two cellular transmembrane BAX inhibitor motif-containing (TMBIM) proteins: Bax inhibitor-1 and Golgi anti-apoptotic protein, both involved in the regulation of cellular Ca^{2+} homeostasis and adaptive cell responses to stress conditions. Thus, alongside pUL36, pUL37x1 and pUL38, pUS21 contributes to maintaining the viability of the host cell until the virus has completed the infection cycle.

A second mechanism used by CMV to counteract ER stress response involves the downregulation of inositol-requiring enzyme 1 (IRE1) protein levels, an ER stress sensor and cell death executor (Maly and Papa, 2014). Misfolded proteins activate IRE1, which in turn oligomerizes and self-activates its RNase activity, leading to degradation of unfolded proteins and upregulation of ER chaperon to enhance protein folding. IRE1 activation also leads to the recruitment of the TNF receptor associated factor (TRAF)-2 and activation of caspase-12 or JNK. Activated JNK induces cells death by activating proapoptotic BH3 proteins while inhibiting the antiapoptotic Bcl-2. Lastly, both MCMV and HCMV homologs M50 and UL50 enhance IRE1 degradation at later times post-infection, thus preventing all IRE1 signaling events (Stahl et al., 2013).

A second form of stress response induced by HCMV infection is that elicited by DNA damage. To ensure faithful duplication and inheritance of genetic material, cells have

evolved mechanisms – collectively termed the DNA-damage response (DDR) – of DNA damage detection to induce DNA repair or, if the damage is too severe, to induce cell death (Xiaofei and Kowalik, 2014). After cell entry, HCMV capsids travel to the nucleus where the linear genome is released and circularized to serve as a template for transcription and replication by a rolling circle mechanism. This process generates multiple exposed ends that can be recognized as dsDNA by activating ataxia-telangiectasia mutated protein (ATM) and rad-3 related kinases (ATR), which initiate the DNA damage signal transduction pathway by targeting proteins involved in the checkpoint response, such as checkpoint kinase 2 (Chk2). In this regard, recent studies have revealed that HCMV can neutralize host DDR at the level of Chk2. In particular, ATM and ChK2 are mislocalized from the nucleus to the cytoplasm where they colocalize with virion structural proteins, which prevents them from initiating the DNA repair process (Gaspar and Shenk, 2006; Luo et al., 2007).

CONCLUSION

Here, we have provided a comprehensive overview of the main characteristics of HCMV that have allowed this virus to evolve multiple immune evasion strategies and achieve latency and seroprevalence. These include the advanced organization and large size of its genome, restricted host specificity, viral latency and sporadic reactivation.

We have also highlighted how the host innate immune response reacts against HCMV infection through different effector cells (e.g., APCs, NK cells, and phagocytes), anti-inflammatory cytokines and IFNs. Briefly, while APCs mediate early immune activation by triggering specific T-cell responses, and cytotoxic NK cells are potent eliminators of HCMV-infected cells, early release of IFN-I and other pro-inflammatory cytokines limit the infection spread through the establishment of the so-called "antiviral state." In addition, several IFN-inducible RFs, which belong to an additional autonomous branch of innate immunity, play a central role in inhibiting viral replication. Lastly, a significant part of the innate immune response is represented by programmed cell death, as apoptotic control greatly contributes to the removal of original population of HCMV-infected cells. Thus, thanks to the presence of multiple innate immune protective mechanisms the host, in most cases, is able to counteract HCMV spread.

However, in the course of host-virus coevolution, as described in this review, HCMV has acquired an extremely wide range of counter-defense mechanisms and manipulation strategies directed against each arm of innate immunity. For instance, HCMV is able to inhibit NK cell activation by encoding numerous proteins targeting multiple host ligands, which are likely to promote viral persistence *in vivo*. The virus is also capable of subverting the immune functions of APCs by reprogramming them as efficient means of viral dissemination, while offsetting their immune surveillance by interfering with MHC-I and MHC-II antigen presentation. Moreover, HCMV can block premature death of infected cells, thereby promoting viral replication. Major interfering with IFN-signaling pathways is also accomplished *via* a wide range of viral proteins that counteract and manipulate IFN production by the host. Thus, there is growing evidence of a highly dynamic and complex interplay between the virus and the IFN system.

From all these data, it is clear that HCMV disease progression depends on the balance between antiviral immune response and viral attempts to manipulate such response to its own advantage. Given the clinical burden of HCMV in immunocompromised patients and congenitally infected infants, there is undoubtedly an urgent and unmet medical need for an effective vaccine against this virus. Significant efforts should also be directed toward the development of more effective therapeutic agents with fewer side effects capable of targeting the virus during both its lytic and latent phases. In this regard, an in-depth analysis of the interplay among HCMV, RFs and INFs resulting in immune evasion should provide potential novel druggable targets.

AUTHOR CONTRIBUTIONS

VD and MD developed the ideas and drafted the manuscript. VD, MB, FG, GGa, and CC wrote sections of the manuscript. GGr, AZ, and SP drew the figures. GGa and MD professionally edited the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This research was supported by the Italian Ministry of Education, University and Research-MIUR (PRIN 2015W729WH) (MD and CC) (PRIN 2015RMNSTA) (VD), the Compagnia San Paolo of Turin (IFIBD) (MD), the University of Turin (RILO1801) (MD) (VD) (FG). GGa and GGr were supported by the European Commission under the Horizon2020 program (H2020 MSCA-ITN-2015).

ACKNOWLEDGMENTS

We thank Dr. Marcello Arsura for editing and proofreading the manuscript.

REFERENCES

Abate, D. A., Watanabe, S., and Mocarski, E. S. (2004). Major human cytomegalovirus structural protein pp65 (ppUL83) prevents interferon response factor 3 activation in the interferon response. *J. Virol.* 78, 10995–11006. doi: 10.1128/JVI.78.20.10995-11006.2004

Adler, M., Tavalai, N., Müller, R., and Stamminger, T. (2011). Human cytomegalovirus immediate-early gene expression is restricted by the nuclear domain 10 component Sp100. *J. Gen. Virol.* 92, 1532–1538. doi: 10.1099/vir.0.030981-0

Ahn, J. H., and Hayward, G. S. (1997). The major immediate-early proteins IE1 and IE2 of human cytomegalovirus colocalize with and disrupt PML-associated

nuclear bodies at very early times in infected permissive cells. *J. Virol.* 71, 4599–4613. doi: 10.1128/jvi.71.6.4599-4613.1997

Akira, S., Uematsu, S., and Takeuchi, O. (2006). Pathogen recognition and innate immunity. *Cell* 124, 783–801. doi: 10.1016/j.cell.2006.02.015

Arase, H., Mocarski, E. S., Campbell, A. E., Hill, A. B., and Lanier, L. L. (2002). Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science* 296, 1323–1326. doi: 10.1126/science.1070884

Ashiru, O., Bennett, N. J., Boyle, L. H., Thomas, M., Trowsdale, J., and Wills, M. R. (2009). NKG2D ligand MICA is retained in the cis-Golgi apparatus by human cytomegalovirus protein UL142. *J. Virol.* 83, 12345–12354. doi: 10.1128/JVI.01175-09

Ashley, C. L., Abendroth, A., McSharry, B. P., and Slobedman, B. (2019). Interferon-independent innate responses to cytomegalovirus. *Front. Immunol.* 10:2751. doi: 10.3389/fimmu.2019.02751

Avdic, S., McSharry, B. P., and Slobedman, B. (2014). Modulation of dendritic cell functions by viral IL-10 encoded by human cytomegalovirus. *Front. Microbiol* 5:337. doi: 10.3389/fmicb.2014.00337

Avdic, S., McSharry, B. P., Steain, M., Poole, E., Sinclair, J., Abendroth, A., et al. (2016). Human cytomegalovirus-encoded human interleukin-10 (IL-10) homolog amplifies its immunomodulatory potential by upregulating human IL-10 in monocytes. *J. Virol.* 90, 3819–3827. doi: 10.1128/JVI.03066-15

Babić, M., Krmpotić, A., and Jonjić, S. (2011). All is fair in virus-host interactions: NK cells and cytomegalovirus. *Trends Mol. Med.* 17, 677–685. doi: 10.1016/j.molmed.2011.07.003

Beck, K., Meyer-König, U., Weidmann, M., Nern, C., and Hufert, F. T. (2003). Human cytomegalovirus impairs dendritic cell function: a novel mechanism of human cytomegalovirus immune escape. *Eur. J. Immunol.* 33, 1528–1538. doi: 10.1002/eji.200323612

Beck, S., and Barrell, B. G. (1988). Human cytomegalovirus encodes a glycoprotein homologous to MHC class-I antigens. *Nature* 331, 269–272. doi: 10.1038/331269a0

Becker, J., Kinast, V., Döring, M., Lipps, C., and Duran, V. (2018). Human monocyte-derived macrophages inhibit HCMV spread independent of classical antiviral cytokines. *Virulence* 9, 669–1684. doi: 10.1080/21505594.2018.1535785

Bennett, N. J., Ashiru, O., Morgan, F. J. E., Pang, Y., Okecha, G., Eagle, R. A., et al. (2010). Intracellular sequestration of the NKG2D ligand ULBP3 by human cytomegalovirus. *J. Immunol.* 185, 1093–1102. doi: 10.4049/jimmunol.1000789

Bhat, T. A., Chaudhary, A. K., Kumar, S., O'Malley, J., Inigo, J. R., Kumar, R., et al. (2017). Endoplasmic reticulum-mediated unfolded protein response and mitochondrial apoptosis in cancer. *Biochim. Biophys. Acta Rev. Cancer* 1867, 58–66. doi: 10.1016/j.bbcan.2016.12.002

Bieniasz, P. D. (2003). Restriction factors: a defense against retroviral infection. *Trends Microbiol.* 11, 286–291. doi: 10.1016/s0966-842x(03)00123-9

Bieniasz, P. D. (2004). Intrinsic immunity: a front-line defense against viral attack. *Nat. Immunol.* 5, 1109–1115. doi: 10.1038/ni1125

Biolatti, M., Dell'Oste, V., De Andrea, M., and Landolfo, S. (2018a). The human cytomegalovirus tegument protein pp65 (pUL83): a key player in innate immune evasion. *New Microbiol.* 41, 87–94.

Biolatti, M., Dell'Oste, V., Pautasso, S., Gugliesi, F., von Einem, J., Krapp, C., et al. (2018b). Human cytomegalovirus tegument protein pp65 (pUL83) dampens type I interferon production by inactivating the DNA sensor cGAS without affecting STING. *J. Virol.* 92:e01774-17. doi: 10.1128/JVI.01774-17

Biolatti, M., Dell'Oste, V., Pautasso, S., von Einem, J., Marschall, M., Plachter, B., et al. (2016). Regulatory interaction between the cellular restriction factor IFI16 and viral pp65 (pUL83) modulates viral gene expression and IFI16 protein stability. *J. Virol.* 90, 8238–8250. doi: 10.1128/JVI.00923-16

Biolatti, M., Gugliesi, F., Dell'Oste, V., and Landolfo, S. (2018c). Modulation of the innate immune response by human cytomegalovirus. *Infect. Genet. Evol.* 64, 105–114. doi: 10.1016/j.meegid.2018.06.025

Biron, C. A., Byron, K. S., and Sullivan, J. L. (1989). Severe herpesvirus infections in an adolescent without natural killer cells. *N. Engl. J. Med.* 320, 1731–1735. doi: 10.1056/NEJM198906293202605

Blanco-Melo, D., Venkatesh, S., and Bieniasz, P. D. (2012). Intrinsic cellular defenses against human immunodeficiency viruses. *Immunity* 37, 399–411. doi: 10.1016/j.immuni.2012.08.013

Bördlein, A., Scherthan, H., Nelkenbrecher, C., Molter, T., Bösl, M. R., Dippold, C., et al. (2011). SPOC1 (PHF13) is required for spermatogonial stem cell differentiation and sustained spermatogenesis. *J. Cell. Sci.* 124, 3137–3148. doi: 10.1242/jcs.085936

Bottino, C., Castriconi, R., Pende, D., Rivera, P., Nanni, M., Carnemolla, B., et al. (2003). Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. *J. Exp. Med.* 198, 557–567. doi: 10.1084/jem.20030788

Braud, V. M., Allan, D. S., O'Callaghan, C. A., Söderström, K., D'Andrea, A., Ogg, G. S., et al. (1998). HLA-E binds to natural killer cell receptors CD94/NKG2A. B and C. *Nature* 391, 795–799. doi: 10.1038/35869

Britt, W. J. (2018). Maternal immunity and the natural history of congenital human cytomegalovirus infection. *Viruses* 10:405. doi: 10.3390/v10080405

Britt, W. J., and Prichard, M. N. (2018). New therapies for human cytomegalovirus infections. *Antiviral Res.* 159, 153–174. doi: 10.1016/j.antiviral.2018.09.003

Brockman, M. A., Kwon, D. S., Tighe, D. P., Pavlik, D. F., Rosato, P. C., Sela, J., et al. (2009). IL-10 is up-regulated in multiple cell types during viremic HIV infection and reversibly inhibits virus-specific T cells. *Blood* 114, 346–356. doi: 10.1182/blood-2008-12-191296

Brooks, A. G., Borrego, F., Posch, P. E., Patamawenu, A., Scorzelli, C. J., Ulbrecht, M., et al. (1999). Specific recognition of HLA-E, but not classical, HLA class I molecules by soluble CD94/NKG2A and NK cells. *J. Immunol.* 162, 305–313.

Brown, M. G., and Scalzo, A. A. (2008). NK gene complex dynamics and selection for NK cell receptors. *Semin. Immunol.* 20, 361–368. doi: 10.1016/j.smim.2008.06.004

Browne, E. P., and Shenk, T. (2003). Human cytomegalovirus UL83-coded pp65 virion protein inhibits antiviral gene expression in infected cells. *Proc. Natl. Acad. Sci. U.S.A.* 100, 11439–11444. doi: 10.1073/pnas.1534570100

Browne, H., Smith, G., Beck, S., and Minson, T. (1990). A complex between the MHC class I homologue encoded by human cytomegalovirus and beta 2 microglobulin. *Nature* 347, 770–772. doi: 10.1038/347770a0

Brune, W., and Andoniou, C. E. (2017). Die another day: inhibition of cell death pathways by cytomegalovirus. *Viruses* 9:249. doi: 10.3390/v9090249

Bukowski, J. F., Woda, B. A., and Welsh, R. M. (1984). Pathogenesis of murine cytomegalovirus infection in natural killer cell-depleted mice. *J. Virol.* 52, 119–128. doi: 10.1128/jvi.52.1.119-128.1984

Cannon, M. J., Schmid, D. S., and Hyde, T. B. (2010). Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. *Rev. Med. Virol.* 20, 202–213. doi: 10.1002/rmv.655

Cantrell, S. R., and Bresnahan, W. A. (2005). Interaction between the human cytomegalovirus UL82 gene product (pp71) and hDaxx regulates immediate-early gene expression and viral replication. *J. Virol.* 79, 7792–7802. doi: 10.1128/JVI.79.12.7792-7802.2005

Cerboni, C., Achour, A., Wärnmark, A., Mousavi-Jazi, M., Sandalova, T., Hsu, M.-L., et al. (2006). Spontaneous mutations in the human CMV HLA class I homologue UL18 affect its binding to the inhibitory receptor LIR-1/ILT2/CD85j. *Eur. J. Immunol.* 36, 732–741. doi: 10.1002/eji.200425220

Cerboni, C., Fionda, C., Soriani, A., Zingoni, A., Doria, M., Cippitelli, M., et al. (2014). The DNA damage response: a common pathway in the regulation of NKG2D and DNAM-1 ligand expression in normal, infected, and cancer cells. *Front. Immunol.* 4:508. doi: 10.3389/fimmu.2013.00508

Cerboni, C., Mousavi-Jazi, M., Linde, A., Söderström, K., Brytting, M., Wahren, B., et al. (2000). Human cytomegalovirus strain-dependent changes in NK cell recognition of infected fibroblasts. *J. Immunol.* 164, 4775–4782. doi: 10.4049/jimmunol.164.9.4775

Chapman, T. L., Heikeman, A. P., and Bjorkman, P. J. (1999). The inhibitory receptor LIR-1 uses a common binding interaction to recognize class I MHC molecules and the viral homolog UL18. *Immunity* 11, 603–613. doi: 10.1016/s1074-7613(00)80135-1

Charpak-Amikam, Y., Kubsch, T., Seidel, E., Oiknine-Djian, E., Cavaletto, N., Yamin, R., et al. (2017). Human cytomegalovirus escapes immune recognition by NK cells through the downregulation of B7-H6 by the viral genes US18 and US20. *Sci. Rep.* 7:8661. doi: 10.1038/s41598-017-08866-2

Chaudhry, M. Z., Kasmapour, B., Plaza-Sirvent, C., Bajagic, M., Casalegno Garduño, R., Borkner, L., et al. (2017). UL36 Rescues Apoptosis Inhibition and In vivo Replication of a Chimeric MCMV Lacking the M36 Gene. *Front. Cell Infect. Microbiol.* 7:312. doi: 10.3389/fcimb.2017.00312

Chin, K. C., and Cresswell, P. (2001). Viperin (cig5), an IFN-inducible antiviral protein directly induced by human cytomegalovirus. *Proc. Natl. Acad. Sci. U.S.A.* 98, 15125–15130. doi: 10.1073/pnas.011593298

Choi, H. J., Park, A., Kang, S., Lee, E., Lee, T. A., Ra, E. A., et al. (2018). Human cytomegalovirus-encoded US9 targets MAVS and STING signaling to evade type I interferon immune responses. *Nat. Commun.* 9:125. doi: 10.1038/s41467-017-02624-8

Chung, H.-R., Xu, C., Fuchs, A., Mund, A., Lange, M., Staege, H., et al. (2016). PHF13 is a molecular reader and transcriptional co-regulator of H3K4me2/3. *eLife* 5:e10607. doi: 10.7554/eLife.10607

Colberg-Poley, A. M., Patel, M. B., Erezo, D. P., and Slater, J. E. (2000). Human cytomegalovirus UL37 immediate-early regulatory proteins traffic through the secretory apparatus and to mitochondria. *J. Gen. Virol.* 81, 1779–1789. doi: 10.1099/0022-1317-81-7-1779

Collin, M., McGovern, N., and Haniffa, M. (2013). Human dendritic cell subsets. *Immunology* 140, 22–30. doi: 10.1111/imm.12117

Collins-McMillen, D., Chesnokova, L., Lee, B.-J., Fulkerson, H. L., Brooks, R., Mosher, B. S., et al. (2018). HCMV infection and apoptosis: how do monocytes survive HCMV infection?. *Viruses* 10:533. doi: 10.3390/v10100533

Compton, T., Kurt-Jones, E. A., Boehme, K. W., Belko, J., Latz, E., Golenbock, D. T., et al. (2003). Human cytomegalovirus activates inflammatory cytokine responses via CD14 and Toll-like receptor 2. *J. Virol.* 77, 4588–4596. doi: 10.1128/jvi.77.8.4588-4596.2003

Cosman, D., Müllberg, J., Sutherland, C. L., Chin, W., Armitage, R., Fanslow, W., et al. (2001). ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity* 14, 123–133. doi: 10.1016/s1074-7613(01)00095-4

Cosme, R. C., Martínez, F. P., and Tang, Q. (2011). Functional interaction of nuclear domain 10 and its components with cytomegalovirus after infections: cross-species host cells versus native cells. *PLoS One* 6:e19187. doi: 10.1371/journal.pone.0019187

Crameri, M., Bauer, M., Caduff, N., Walker, R., Steiner, F., Franzoso, F. D., et al. (2018). MxB is an interferon-induced restriction factor of human herpesviruses. *Nat. Commun.* 9:1980. doi: 10.1038/s41467-018-04379-2

Cristea, I. M., Moorman, N. J., Terhune, S. S., Cuevas, C. D., O'Keefe, E. S., Rout, M. P., et al. (2010). Human cytomegalovirus pUL83 stimulates activity of the viral immediate-early promoter through its interaction with the cellular IFI16 protein. *J. Virol.* 84, 7803–7814. doi: 10.1128/JVI.00139-10

Dell'Oste, V., Gatti, D., Gugliesi, F., De Andrea, M., Bawadekar, M., Lo Cigno, I., et al. (2014). Innate nuclear sensor IFI16 translocates into the cytoplasm during the early stage of in vitro human cytomegalovirus infection and is entrapped in the egressing virions during the late stage. *J. Virol.* 88, 6970–6982. doi: 10.1128/JVI.00384-14

Diner, B. A., Lum, K. K., Toettcher, J. E., and Cristea, I. M. (2016). Viral DNA Sensors IFI16 and Cyclic GMP-AMP synthase possess distinct functions in regulating viral gene expression, immune defenses, and apoptotic responses during herpesvirus infection. *mBio* 7:e01553-16. doi: 10.1128/mBio.01553-16

Duggal, N. K., and Emerman, M. (2012). Evolutionary conflicts between viruses and restriction factors shape immunity. *Nat. Rev. Immunol.* 12, 687–695. doi: 10.1038/nri3295

Dunn, C., Chalupny, N. J., Sutherland, C. L., Dosch, S., Sivakumar, P. V., Johnson, D. C., et al. (2003). Human cytomegalovirus glycoprotein UL16 causes intracellular sequestration of NKG2D ligands, protecting against natural killer cell cytotoxicity. *J. Exp. Med.* 197, 1427–1439. doi: 10.1084/jem.20022059

Eagle, R. A., Traherne, J. A., Hair, J. R., Jafferji, I., and Trowsdale, J. (2009). ULBP6/RAET1L is an additional human NKG2D ligand. *Eur. J. Immunol.* 39, 3207–3216. doi: 10.1002/eji.200939502

Elder, E. G., Krishna, B. A., Williamson, J., Lim, E. Y., and Poole, E. (2019). Interferon-responsive genes are targeted during the establishment of human cytomegalovirus latency. *mBio* 10:e02574-19. doi: 10.1128/mBio.02574-19

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicol. Pathol.* 35, 495–516. doi: 10.1080/01926230701320337

Estaquier, J., Vallette, F., Vayssiere, J.-L., and Mignotte, B. (2012). The mitochondrial pathways of apoptosis. *Adv. Exp. Med. Biol.* 942, 157–183. doi: 10.1007/978-94-007-2869-1_7

Farrell, H. E., Bruce, K., Ma, J., Davis-Poynter, N., and Stevenson, P. G. (2018). Human cytomegalovirus US28 allows dendritic cell exit from lymph nodes. *J. Gen. Virol.* 99, 1509–1514. doi: 10.1099/jgv.0.001154

Feng, L., Sheng, J., Vu, G.-P., Liu, Y., Foo, C., Wu, S., et al. (2018). Human cytomegalovirus UL23 inhibits transcription of interferon-γ stimulated genes and blocks antiviral interferon-γ responses by interacting with human N-myc interactor protein. *PLoS Pathog.* 14:e1006867. doi: 10.1371/journal.ppat.1006867

Fielding, C. A., Weekes, M. P., Nobre, L. V., Ruckova, E., Wilkie, G. S., Paulo, J. A., et al. (2017). Control of immune ligands by members of a cytomegalovirus gene expansion suppresses natural killer cell activation. *eLife* 6:e22206. doi: 10.7554/eLife.22206

Frascaroli, G., Lecher, C., Varani, S., Setz, C., van der Merwe, J., Brune, W., et al. (2018). Human macrophages escape inhibition of major histocompatibility complex-dependent antigen presentation by cytomegalovirus and drive proliferation and activation of memory CD4+ and CD8+ T cells. *Front. Immunol.* 9:1129. doi: 10.3389/fimmu.2018.01129

Frascaroli, G., Varani, S., Blankenhorn, N., Pretsch, R., Bacher, M., Leng, L., et al. (2009). Human cytomegalovirus paralyzes macrophage motility through down-regulation of chemokine receptors, reorganization of the cytoskeleton, and release of macrophage migration inhibitory factor. *J. Immunol.* 182, 477–488. doi: 10.4049/jimmunol.182.1.477

Frascaroli, G., Varani, S., Moepps, B., Sinzger, C., Landini, M. P., and Mertens, T. (2006). Human cytomegalovirus subverts the functions of monocytes, impairing chemokine-mediated migration and leukocyte recruitment. *J. Virol.* 80, 7578–7589. doi: 10.1128/JVI.02421-05

Fu, Y.-Z., Su, S., Gao, Y.-Q., Wang, P.-P., Huang, Z.-F., Hu, M.-M., et al. (2017). Human cytomegalovirus tegument protein UL82 inhibits STING-mediated signaling to evade antiviral immunity. *Cell Host Microbe* 21, 231–243. doi: 10.1016/j.chom.2017.01.001

Gafa, V., Manches, O., Pastor, A., Drouet, E., Ambroise-Thomas, P., Grillot, R., et al. (2005). Human cytomegalovirus downregulates complement receptors (CR3, CR4) and decreases phagocytosis by macrophages. *J. Med. Virol.* 76, 361–366. doi: 10.1002/jmv.20358

Gariano, G. R., Dell'Oste, V., Bronzini, M., Gatti, D., Luganini, A., De Andrea, M., et al. (2012). The intracellular DNA sensor IFI16 gene acts as restriction factor for human cytomegalovirus replication. *PLoS Pathog.* 8:e1002498. doi: 10.1371/journal.ppat.1002498

Gaspar, M., and Shenk, T. (2006). Human cytomegalovirus inhibits a DNA damage response by mislocalizing checkpoint proteins. *Proc. Natl. Acad. Sci. U.S.A.* 103, 2821–2826. doi: 10.1073/pnas.0511148103

Glass, M., and Everett, R. D. (2013). Components of promyelocytic leukemia nuclear bodies (ND10) act cooperatively to repress herpesvirus infection. *J. Virol.* 87, 2174–2185. doi: 10.1128/JVI.02950-12

Goldmacher, V. S., Bartle, L. M., Skaletskaya, A., Dionne, C. A., Kedersha, N. L., Vater, C. A., et al. (1999). A cytomegalovirus-encoded mitochondria-localized inhibitor of apoptosis structurally unrelated to Bcl-2. *Proc. Natl. Acad. Sci. U.S.A.* 96, 12536–12541. doi: 10.1073/pnas.96.22.12536

Goodier, M. R., Jonjić, S., Riley, E. M., and Juranić Lisnić, V. (2018). CMV and natural killer cells: shaping the response to vaccination. *Eur. J. Immunol.* 48, 50–65. doi: 10.1002/eji.201646762

Goodwin, C. M., Ciesla, J. H., and Munger, J. (2018). Who's driving? Human cytomegalovirus, interferon, and NFκB signaling. *Viruses* 10:447. doi: 10.3390/v10090447

Goodwin, C. M., and Munger, J. (2019). The IκB kinases restrict human cytomegalovirus infection. *J. Virol.* 93:e02030-18. doi: 10.1128/JVI.02030-18

Gredmark, S., Britt, W. B., Xie, X., Lindbom, L., and Söderberg-Nauclér, C. (2004). Human cytomegalovirus induces inhibition of macrophage differentiation by binding to human aminopeptidase N/CD13. *J. Immunol.* 173, 4897–4907. doi: 10.4049/jimmunol.173.8.4897

Gredmark, S., and Söderberg-Nauclér, C. (2003). Human cytomegalovirus inhibits differentiation of monocytes into dendritic cells with the consequence of depressed immunological functions. *J. Virol.* 77, 10943–10956. doi: 10.1128/jvi.77.20.10943-10956.2003

Green, D. R. (2019). The coming decade of cell death research: five riddles. *Cell* 177, 1094–1107. doi: 10.1016/j.cell.2019.04.024

Griffiths, P., Baraniak, I., and Reeves, M. (2015). The pathogenesis of human cytomegalovirus. *J. Pathol.* 235, 288–297. doi: 10.1002/path.4437

Gumá, M., Angulo, A., Vilches, C., Gómez-Lozano, N., Malats, N., and López-Botet, M. (2004). Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. *Blood* 104, 3664–3671. doi: 10.1182/blood-2004-05-2058

Haller, O., and Kochs, G. (2011). Human MxA protein: an interferon-induced dynamin-like GTPase with broad antiviral activity. *J. Interferon Cytokine Res.* 31, 79–87. doi: 10.1089/jir.2010.0076

Hammer, Q., Rückert, T., and Romagnani, C. (2018). Natural killer cell specificity for viral infections. *Nat. Immunol.* 19, 800–808. doi: 10.1038/s41590-018-0163-6

Harris, R. S., and Dudley, J. P. (2015). APOBECs and virus restriction. *Virology* 479, 131–145. doi: 10.1016/j.virol.2015.03.012

Hertel, L., Lacaille, V. G., Strobl, H., Mellins, E. D., and Mocarski, E. S. (2003). Susceptibility of immature and mature Langerhans cell-type dendritic cells to infection and immunomodulation by human cytomegalovirus. *J. Virol.* 77, 7563–7574. doi: 10.1128/jvi.77.13.7563-7574.2003

Hofmann, H., Sindre, H., and Stamminger, T. (2002). Functional interaction between the pp71 protein of human cytomegalovirus and the PML-interacting protein human Daxx. *J. Virol.* 76, 5769–5783. doi: 10.1128/jvi.76.11.5769-5783.2002

Hofmann, S., Dehn, S., Businger, R., Bolduan, S., Schneider, M., Debyser, Z., et al. (2017). Dual role of the chromatin-binding factor PHF13 in the pre- and post-integration phases of HIV-1 replication. *Open Biol.* 7:170115. doi: 10.1098/rsob.170115

Hotter, D., and Kirchhoff, F. (2018). Interferons and beyond: induction of antiretroviral restriction factors. *J. Leukoc. Biol.* 103, 465–477. doi: 10.1002/JLB.3MR0717-307R

Hsu, J.-L., Chen, W.-H., Bai, C.-H., Leu, J.-G., Hsu, C.-Y., Viergever, M. A., et al. (2015). Microstructural white matter tissue characteristics are modulated by homocysteine: a diffusion tensor imaging study. *PLoS One* 10:e0116330. doi: 10.1371/journal.pone.0116330

Huang, Z.-F., Zou, H.-M., Liao, B.-W., Zhang, H.-Y., Yang, Y., Fu, Y.-Z., et al. (2018). Human cytomegalovirus protein UL31 inhibits DNA sensing of cGAS to mediate immune evasion. *Cell Host Microbe* 24, 69.e4–80.e4. doi: 10.1016/j.chom.2018.05.007

Iguchi-Manaka, A., Kai, H., Yamashita, Y., Shibata, K., Tahara-Hanaoka, S., Honda, S., et al. (2008). Accelerated tumor growth in mice deficient in DNAM-1 receptor. *J. Exp. Med.* 205, 2959–2964. doi: 10.1084/jem.20081611

Iwanaszko, M., and Kimmel, M. (2015). NF-κB and IRF pathways: cross-regulation on target genes promoter level. *BMC Genomics* 16:307. doi: 10.1186/s12864-015-1511-7

Jackson, J. W., and Sparer, T. (2018). There is always another way! cytomegalovirus' multifaceted dissemination schemes. *Viruses* 10:383. doi: 10.3390/v10070383

Jahn, G., Stenglein, S., Riegler, S., Einsele, H., and Sinzger, C. (1999). Human cytomegalovirus infection of immature dendritic cells and macrophages. *Intervirology* 42, 365–372. doi: 10.1159/000053973

Jønsson, K. L., Laustsen, A., Krapp, C., Skipper, K. A., Thavachelvam, K., Hotter, D., et al. (2017). IFI16 is required for DNA sensing in human macrophages by promoting production and function of cGAMP. *Nat. Commun.* 8:14391. doi: 10.1038/ncomms14391

Kaarbø, M., Ager-Wick, E., Osenbroch, P. Ø, Kilander, A., Skinnes, R., Müller, F., et al. (2011). Human cytomegalovirus infection increases mitochondrial biogenesis. *Mitochondrion* 11, 935–945. doi: 10.1016/j.mito.2011.08.008

Kasmapour, B., Kubsch, T., Rand, U., Eiz-Vesper, B., Messerle, M., Vondran, F. W. R., et al. (2017). Myeloid dendritic cells repress human cytomegalovirus gene expression and spread by releasing interferon-unrelated soluble antiviral factors. *J. Virol.* 92:e01138-17. doi: 10.1128/JVI.01138-17

Kim, J.-E., Kim, Y.-E., Stinski, M. F., Ahn, J.-H., and Song, Y.-J. (2017). Human Cytomegalovirus IE2 86 kDa Protein Induces STING Degradation and Inhibits cGAMP-mediated IFN-β induction. *Front. Microbiol.* 8:1854. doi: 10.3389/fmicb.2017.01854

Kinkley, S., Staege, H., Mohrmann, G., Rohaly, G., Schaub, T., Kremmer, E., et al. (2009). SPOC1: a novel PHD-containing protein modulating chromatin structure and mitotic chromosome condensation. *J. Cell. Sci.* 122, 2946–2956. doi: 10.1242/jcs.047365

Korioth, F., Maul, G. G., Plachter, B., Stamminger, T., and Frey, J. (1996). The nuclear domain 10 (ND10) is disrupted by the human cytomegalovirus gene product IE1. *Exp. Cell Res.* 229, 155–158. doi: 10.1006/excr.1996.0353

Kruidering, M., and Evan, G. I. (2000). Caspase-8 in apoptosis: the beginning of "the end"? *IUBMB Life* 50, 85–90. doi: 10.1080/713803693

Kubin, M., Cassiano, L., Chalupny, J., Chin, W., Cosman, D., Fanslow, W., et al. (2001). ULBP1, 2, 3: novel MHC class I-related molecules that bind to human cytomegalovirus glycoprotein UL16, activate NK cells. *Eur. J. Immunol.* 31, 1428–1437. doi: 10.1002/1521-4141(200105)31:5<1428::aid-immu1428>3.0.co;2-4

Lanier, L. L. (2015). NKG2D receptor and its ligands in host defense. *Cancer Immunol. Res.* 3, 575–582. doi: 10.1158/2326-6066.CIR-15-0098

Lee, H.-R., Kim, D.-J., Lee, J.-M., Choi, C. Y., Ahn, B.-Y., Hayward, G. S., et al. (2004). Ability of the human cytomegalovirus IE1 protein to modulate sumoylation of PML correlates with its functional activities in transcriptional regulation and infectivity in cultured fibroblast cells. *J. Virol.* 78, 6527–6542. doi: 10.1128/JVI.78.12.6527-6542.2004

Lee, N., Llano, M., Carretero, M., Ishitani, A., Navarro, F., López-Botet, M., et al. (1998). HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc. Natl. Acad. Sci. U.S.A.* 95, 5199–5204. doi: 10.1073/pnas.95.9.5199

Leitner, J., Herndler-Brandstetter, D., Zlabinger, G. J., Grubeck-Loebenstein, B., and Steinberger, P. (2015). CD58/CD2 is the primary costimulatory pathway in human CD28-CD8+ T cells. *J. Immunol.* 195, 477–487. doi: 10.4049/jimmunol.1401917

Liu, L. L., Brumbaugh, J., Bar-Nur, O., Smith, Z., Stadtfeld, M., Meissner, A., et al. (2016). Probabilistic modeling of reprogramming to induced pluripotent stem cells. *Cell Rep.* 17, 3395–3406. doi: 10.1016/j.celrep.2016.11.080

Loewendorf, A., and Benedict, C. A. (2010). Modulation of host innate and adaptive immune defenses by cytomegalovirus: timing is everything. *J. Intern. Med.* 267, 483–501. doi: 10.1111/j.1365-2796.2010.02220.x

López-Botet, M., Muntasell, A., and Vilches, C. (2014). The CD94/NKG2C+ NK-cell subset on the edge of innate and adaptive immunity to human cytomegalovirus infection. *Semin. Immunol.* 26, 145–151. doi: 10.1016/j.smim.2014.03.002

Loureiro, J., and Ploegh, H. L. (2006). Antigen presentation and the ubiquitin-proteasome system in host-pathogen interactions. *Adv. Immunol.* 92, 225–305. doi: 10.1016/S0065-2776(06)92006-9

Luecke, S., and Paludan, S. R. (2015). Innate recognition of alphaherpesvirus DNA. *Adv. Virus Res.* 92, 63–100. doi: 10.1016/bs.aivir.2014.11.003

Luganini, A., Di Nardo, G., Munaron, L., Gilardi, G., Fiorio Pla, A., and Gribaudo, G. (2018). Human cytomegalovirus US21 protein is a viroporin that modulates calcium homeostasis and protects cells against apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* 115, E12370–E12377. doi: 10.1073/pnas.1813183115

Lukashchuk, V., McFarlane, S., Everett, R. D., and Preston, C. M. (2008). Human cytomegalovirus protein pp71 displaces the chromatin-associated factor ATRX from nuclear domain 10 at early stages of infection. *J. Virol.* 82, 12543–12554. doi: 10.1128/JVI.01215-08

Luo, M. H., Rosenke, K., Czornak, K., and Fortunato, E. A. (2007). Human cytomegalovirus disrupts both ataxia telangiectasia mutated protein (ATM)- and ATM-Rad3-related kinase-mediated DNA damage responses during lytic infection. *J. Virol.* 81, 1934–1950. doi: 10.1128/JVI.01670-06

Mace, E. M., and Orange, J. S. (2019). Emerging insights into human health and NK cell biology from the study of NK cell deficiencies. *Immunol. Rev.* 287, 202–225. doi: 10.1111/imr.12725

Machala, E. A., Avdic, S., Stern, L., Zajonc, D. M., Benedict, C. A., Blyth, E., et al. (2019). Restriction of human cytomegalovirus infection by galectin-9. *J. Virol.* 93:e01746-18. doi: 10.1128/JVI.01746-18

Maly, D. J., and Papa, F. R. (2014). Druggable sensors of the unfolded protein response. *Nat. Chem. Biol.* 10, 892–901. doi: 10.1038/nchembio.1664

Manicklal, S., Emery, V. C., Lazzarotto, T., Boppana, S. B., and Gupta, R. K. (2013). The "silent" global burden of congenital cytomegalovirus. *Clin. Microbiol. Rev.* 26, 86–102. doi: 10.1128/CMR.00062-12

Marques, M., Ferreira, A. R., and Ribeiro, D. (2018). The interplay between human cytomegalovirus and pathogen recognition receptor signaling. *Viruses* 10:514. doi: 10.3390/v10100514

McCormick, A. L., Roback, L., Livingston-Rosanoff, D., and St Clair, C. (2010). The human cytomegalovirus UL36 gene controls caspase-dependent and -independent cell death programs activated by infection of monocytes differentiating to macrophages. *J. Virol.* 84, 5108–5123. doi: 10.1128/JVI.01345-09

Merani, S., Chen, W., and Elahi, S. (2015). The bitter side of sweet: the role of Galectin-9 in immunopathogenesis of viral infections. *Rev. Med. Virol.* 25, 175–186. doi: 10.1002/rmv.1832

Mesev, E. V., LeDesma, R. A., and Ploss, A. (2019). Decoding type I and III interferon signalling during viral infection. *Nat Microbiol.* 4, 914–924. doi: 10.1038/s41564-019-0421-x

Mezger, M., Bonin, M., Kessler, T., Gebhardt, F., Einsele, H., and Loeffler, J. (2009). Toll-like receptor 3 has no critical role during early immune response of human monocyte-derived dendritic cells after infection with the human cytomegalovirus strain TB40E. *Viral Immunol.* 22, 343–351. doi: 10.1089/vim.2009.0011

Mohrmann, G., Hengstler, J. G., Hofmann, T. G., Endele, S. U., Lee, B., Stelzer, C., et al. (2005). SPOC1, a novel PHD-finger protein: association with residual disease and survival in ovarian cancer. *Int. J. Cancer* 116, 547–554. doi: 10.1002/ijc.20912

Moutaftsi, M., Brennan, P., Spector, S. A., and Tabi, Z. (2004). Impaired lymphoid chemokine-mediated migration due to a block on the chemokine receptor switch in human cytomegalovirus-infected dendritic cells. *J. Virol.* 78, 3046–3054. doi: 10.1128/jvi.78.6.3046-3054.2004

Mund, A., Schubert, T., Staege, H., Kinkley, S., Reumann, K., Kriegs, M., et al. (2012). SPOC1 modulates DNA repair by regulating key determinants of chromatin compaction and DNA damage response. *Nucleic Acids Res.* 40, 11363–11379. doi: 10.1093/nar/gks868

Nemčovičová, I., Benedict, C. A., and Zajonc, D. M. (2013). Structure of human cytomegalovirus UL141 binding to TRAIL-R2 reveals novel, non-canonical death receptor interactions. *PLoS Pathog.* 9:e1003224. doi: 10.1371/journal.ppat.1003224

Netea, M. G., Joosten, L. A. B., Latz, E., Mills, K. H. G., Natoli, G., Stunnenberg, H. G., et al. (2016). Trained immunity: a program of innate immune memory in health and disease. *Science* 352:aaf1098. doi: 10.1126/science.aaf1098

Nikzad, R., Angelo, L. S., Aviles-Padilla, K., Le, D. T., Singh, V. K., Bimler, L., et al. (2019). Human natural killer cells mediate adaptive immunity to viral antigens. *Sci. Immunol.* 4:eaat8116. doi: 10.1126/sciimmunol.aat8116

Noyola, D. E., Fortuny, C., Muntasell, A., Noguera-Julian, A., Muñoz-Almagro, C., Alarcón, A., et al. (2012). Influence of congenital human cytomegalovirus infection and the NKG2C genotype on NK-cell subset distribution in children. *Eur. J. Immunol.* 42, 3256–3266. doi: 10.1002/eji.201242752

Odeberg, J., Plachter, B., Brandén, L., and Söderberg-Nauclér, C. (2003). Human cytomegalovirus protein pp65 mediates accumulation of HLA-DR in lysosomes and destruction of the HLA-DR alpha-chain. *Blood* 101, 4870–4877. doi: 10.1182/blood-2002-05-1504

Orange, J. S. (2013). Natural killer cell deficiency. *J. Allergy Clin. Immunol.* 132, 515–525. doi: 10.1016/j.jaci.2013.07.020

O'Sullivan, T. E., Sun, J. C., and Lanier, L. L. (2015). Natural killer cell memory. *Immunity* 43, 634–645. doi: 10.1016/j.immuni.2015.09.013

Paijo, J., Döring, M., Spanier, J., Grabski, E., Nooruzzaman, M., Schmidt, T., et al. (2016). cGAS senses human cytomegalovirus and induces type I interferon responses in human monocyte-derived cells. *PLoS Pathog.* 12:e1005546. doi: 10.1371/journal.ppat.1005546

Paludan, S. R., Bowie, A. G., Horan, K. A., and Fitzgerald, K. A. (2011). Recognition of herpesviruses by the innate immune system. *Nat. Rev. Immunol.* 11, 143–154. doi: 10.1038/nri2937

Patel, M., Vlahava, V.-M., Forbes, S. K., Fielding, C. A., Stanton, R. J., and Wang, E. C. Y. (2018). HCMV-encoded NK modulators: lessons from *in vitro* and *in vivo* genetic variation. *Front. Immunol.* 9:2214. doi: 10.3389/fimmu.2018.02214

Pautasso, S., Galitska, G., Dell'Oste, V., Biolatti, M., Cagliani, R., Forni, D., et al. (2018). Strategy of human cytomegalovirus to escape interferon beta-induced APOBEC3G editing activity. *J. Virol.* 92:e01224-18. doi: 10.1128/JVI.01224-18

Pignoloni, B., Fionda, C., Dell'Oste, V., Luganini, A., Cippitelli, M., Zingoni, A., et al. (2016). Distinct roles for human cytomegalovirus immediate early proteins IE1 and IE2 in the transcriptional regulation of MICA and PVR/CD155 expression. *J. Immunol.* 197, 4066–4078. doi: 10.4049/jimmunol.1502527

Piret, J., and Boivin, G. (2019). Clinical development of letermovir and maribavir: overview of human cytomegalovirus drug resistance. *Antiviral Res.* 163, 91–105. doi: 10.1016/j.antiviral.2019.01.011

Poole, E., Kuan, W. L., Barker, R., and Sinclair, J. (2016). The human cytomegalovirus non-coding Beta2.7 RNA as a novel therapeutic for Parkinson's disease–Translational research with no translation. *Virus Res.* 212, 64–69. doi: 10.1016/j.virusres.2015.05.007

Poole, E. L., Kew, V. G., Lau, J. C. H., Murray, M. J., Stamminger, T., Sinclair, J. H., et al. (2018). A virally encoded deSUMOylase activity is required for cytomegalovirus reactivation from latency. *Cell Rep.* 24, 594–606. doi: 10.1016/j.celrep.2018.06.048

Preston, C. M., and Nicholl, M. J. (2006). Role of the cellular protein hDaxx in human cytomegalovirus immediate-early gene expression. *J. Gen. Virol.* 87, 1113–1121. doi: 10.1099/vir.0.81566-0

Prod'homme, V., Griffin, C., Aicheler, R. J., Wang, E. C. Y., McSharry, B. P., Rickards, C. R., et al. (2007). The human cytomegalovirus MHC class I homolog UL18 inhibits LIR-1+ but activates LIR-1- NK cells. *J. Immunol.* 178, 4473–4481. doi: 10.4049/jimmunol.178.7.4473

Quinnan, G. V., Kirmani, N., Rook, A. H., Manischewitz, J. F., Jackson, L., Moreschi, G., et al. (1982). Cytotoxic t cells in cytomegalovirus infection: HLA-restricted T-lymphocyte and non-T-lymphocyte cytotoxic responses correlate with recovery from cytomegalovirus infection in bone-marrow-transplant recipients. *N. Engl. J. Med.* 307, 7–13. doi: 10.1056/NEJM198207013070102

Rabinovich, G. A., Liu, F.-T., Hirashima, M., and Anderson, A. (2007). An emerging role for galectins in tuning the immune response: lessons from experimental models of inflammatory disease, autoimmunity and cancer. *Scand. J. Immunol.* 66, 143–158. doi: 10.1111/j.1365-3083.2007.01986.x

Rabinovich, G. A., and Toscano, M. A. (2009). Turning "sweet" on immunity: galectin-glycan interactions in immune tolerance and inflammation. *Nat. Rev. Immunol.* 9, 338–352. doi: 10.1038/nri2536

Radosavljevic, M., Cuillerier, B., Wilson, M. J., Clément, O., Wicker, S., Gilfillan, S., et al. (2002). A cluster of ten novel MHC class I related genes on human chromosome 6q24.2-*q*25.3. *Genomics* 79, 114–123. doi: 10.1006/geno.2001.6673

Raftery, M. J., Hitzler, M., Winau, F., Giese, T., Plachter, B., Kaufmann, S. H. E., et al. (2008). Inhibition of CD1 antigen presentation by human cytomegalovirus. *J. Virol.* 82, 4308–4319. doi: 10.1128/JVI.01447-07

Raftery, M. J., Wieland, D., Gronewald, S., Kraus, A. A., Giese, T., and Schönrich, G. (2004). Shaping phenotype, function, and survival of dendritic cells by cytomegalovirus-encoded IL-10. *J. Immunol.* 173, 3383–3391. doi: 10.4049/jimmunol.173.5.3383

Reboredo, M., Greaves, R. F., and Hahn, G. (2004). Human cytomegalovirus proteins encoded by UL37 exon 1 protect infected fibroblasts against virus-induced apoptosis and are required for efficient virus replication. *J. Gen. Virol.* 85, 3555–3567. doi: 10.1099/vir.0.80379-0

Reeves, M. B., and Compton, T. (2011). Inhibition of inflammatory interleukin-6 activity via extracellular signal-regulated kinase-mitogen-activated protein kinase signaling antagonizes human cytomegalovirus reactivation from dendritic cells. *J. Virol.* 85, 12750–12758. doi: 10.1128/JVI.05878-11

Reeves, M. B., Lehner, P. J., Sissons, J. G. P., and Sinclair, J. H. (2005). An in vitro model for the regulation of human cytomegalovirus latency and reactivation in dendritic cells by chromatin remodelling. *J. Gen. Virol.* 86, 2949–2954. doi: 10.1099/vir.0.81161-0

Reeves, M. B., and Sinclair, J. H. (2013). Circulating dendritic cells isolated from healthy seropositive donors are sites of human cytomegalovirus reactivation in vivo. *J. Virol.* 87, 10660–10667. doi: 10.1128/JVI.01539-13

Reichel, A., Stilp, A.-C., Scherer, M., Reuter, N., Lukassen, S., Kasmapour, B., et al. (2018). Chromatin-remodeling factor SPOC1 acts as a cellular restriction factor against human cytomegalovirus by repressing the major immediate early promoter. *J. Virol.* 92:e00342-18. doi: 10.1128/JVI.00342-18

Reiser, M., Marousis, C. G., Nelson, D. R., Lauer, G., González-Peralta, R. P., Davis, G. L., et al. (1997). Serum interleukin 4 and interleukin 10 levels in patients with chronic hepatitis C virus infection. *J. Hepatol.* 26, 471–478. doi: 10.1016/s0168-8278(97)80409-6

Renneson, J., Dutta, B., Goriely, S., Danis, B., Lecomte, S., Laes, J.-F., et al. (2009). IL-12 and type I IFN response of neonatal myeloid DC to human CMV infection. *Eur. J. Immunol.* 39, 2789–2799. doi: 10.1002/eji.200939414

Reuter, N., Schilling, E.-M., Scherer, M., Müller, R., and Stamminger, T. (2017). The ND10 component promyelocytic leukemia protein acts as an E3 ligase for SUMOylation of the major immediate early protein IE1 of human cytomegalovirus. *J. Virol.* 91:e02335-16. doi: 10.1128/JVI.02335-16

Riegler, S., Hebart, H., Einsele, H., Brossart, P., Jahn, G., and Sinzger, C. (2000). Monocyte-derived dendritic cells are permissive to the complete replicative cycle of human cytomegalovirus. *J. Gen. Virol.* 81, 393–399. doi: 10.1099/0022-1317-81-2-393

Rölle, A., and Brodin, P. (2016). Immune adaptation to environmental influence: the case of NK Cells and HCMV. *Trends Immunol.* 37, 233–243. doi: 10.1016/j.it.2016.01.005

Rölle, A., Mousavi-Jazi, M., Eriksson, M., Odeberg, J., Söderberg-Nauclér, C., Cosman, D., et al. (2003). Effects of human cytomegalovirus infection on ligands for the activating NKG2D receptor of NK cells: up-regulation of UL16-binding protein (ULBP)1 and ULBP2 is counteracted by the viral UL16 protein. *J. Immunol.* 171, 902–908. doi: 10.4049/jimmunol.171.2.902

Rossini, G., Cerboni, C., Santoni, A., Landini, M. P., Landolfo, S., Gatti, D., et al. (2012). Interplay between human cytomegalovirus and intrinsic/innate host responses: a complex bidirectional relationship. *Mediators Inflamm.* 2012:607276. doi: 10.1155/2012/607276

Saffert, R. T., and Kalejta, R. F. (2006). Inactivating a cellular intrinsic immune defense mediated by Daxx is the mechanism through which the human cytomegalovirus pp71 protein stimulates viral immediate-early gene expression. *J. Virol.* 80, 3863–3871. doi: 10.1128/JVI.80.8.3863-3871.2006

Salsman, J., Wang, X., and Frappier, L. (2011). Nuclear body formation and PML body remodeling by the human cytomegalovirus protein UL35. *Virology* 414, 119–129. doi: 10.1016/j.virol.2011.03.013

Schierling, K., Stamminger, T., Mertens, T., and Winkler, M. (2004). Human cytomegalovirus tegument proteins ppUL82 (pp71) and ppUL35 interact and cooperatively activate the major immediate-early enhancer. *J. Virol.* 78, 9512–9523. doi: 10.1128/JVI.78.17.9512-9523.2004

Schilling, E.-M., Scherer, M., Reuter, N., Schweininger, J., Muller, Y. A., and Stamminger, T. (2017). The human cytomegalovirus IE1 protein antagonizes PML nuclear body-mediated intrinsic immunity via the inhibition of PML De Novo SUMOylation. *J. Virol.* 91:e02049-16. doi: 10.1128/JVI.02049-16

Schilling, M., Bulli, L., Weigang, S., Graf, L., Naumann, S., Patzina, C., et al. (2018). Human MxB protein is a pan-herpesvirus restriction factor. *J. Virol.* 92:e01056-18. doi: 10.1128/JVI.01056-18

Schleiss, M. R., Permar, S. R., and Plotkin, S. A. (2017). Progress toward development of a vaccine against congenital cytomegalovirus infection. *Clin. Vaccine Immunol.* 24:e00268-17. doi: 10.1128/CVI.00268-17

Schmiedel, D., and Mandelboim, O. (2017). Disarming cellular alarm systems-manipulation of stress-induced NKG2D ligands by human herpesviruses. *Front. Immunol.* 8:390. doi: 10.3389/fimmu.2017.00390

Schneider, K., Meyer-Koenig, U., and Hufert, F. T. (2008). Human cytomegalovirus impairs the function of plasmacytoid dendritic cells in lymphoid organs. *PLoS One* 3:e3482. doi: 10.1371/journal.pone.0003482

Schreiner, S., Kinkley, S., Bürck, C., Mund, A., Wimmer, P., Schubert, T., et al. (2013). SPOC1-mediated antiviral host cell response is antagonized early in human adenovirus type 5 infection. *PLoS Pathog.* 9:e1003775. doi: 10.1371/journal.ppat.1003775

Selvaraj, P., Plunkett, M. L., Dustin, M., Sanders, M. E., Shaw, S., and Springer, T. A. (1987). The T lymphocyte glycoprotein CD2 binds the cell surface ligand LFA-3. *Nature* 326, 400–403. doi: 10.1038/326400a0

Sénéchal, B., Boruchov, A. M., Reagan, J. L., Hart, D. N. J., and Young, J. W. (2004). Infection of mature monocyte-derived dendritic cells with human cytomegalovirus inhibits stimulation of T-cell proliferation via the release of soluble CD83. *Blood* 103, 4207–4215. doi: 10.1182/blood-2003-12-4350

Seo, J.-Y., and Cresswell, P. (2013). Viperin regulates cellular lipid metabolism during human cytomegalovirus infection. *PLoS Pathog.* 9:e1003497. doi: 10.1371/journal.ppat.1003497

Seo, J.-Y., Yaneva, R., Hinson, E. R., and Cresswell, P. (2011). Human cytomegalovirus directly induces the antiviral protein viperin to enhance infectivity. *Science* 332, 1093–1097. doi: 10.1126/science.1202007

Sharon-Friling, R., Goodhouse, J., Colberg-Poley, A. M., and Shenk, T. (2006). Human cytomegalovirus pUL37x1 induces the release of endoplasmic reticulum calcium stores. *Proc. Natl. Acad. Sci. U.S.A.* 103, 19117–19122. doi: 10.1073/pnas.0609353103

Sharon-Friling, R., and Shenk, T. (2014). Human cytomegalovirus pUL37x1-induced calcium flux activates PKCα, inducing altered cell shape and accumulation of cytoplasmic vesicles. *Proc. Natl. Acad. Sci. U.S.A.* 111, E1140–E1148. doi: 10.1073/pnas.1402515111

Shibuya, A., Campbell, D., Hannum, C., Yssel, H., Franz-Bacon, K., McClanahan, T., et al. (1996). DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes. *Immunity* 4, 573–581. doi: 10.1016/s1074-7613(00)70060-4

Shin, H. J., Kim, Y.-E., Kim, E. T., and Ahn, J.-H. (2012). The chromatin-tethering domain of human cytomegalovirus immediate-early (IE) 1 mediates associations of IE1, PML and STAT2 with mitotic chromosomes, but is not essential for viral replication. *J. Gen. Virol.* 93, 716–721. doi: 10.1099/vir.0.037986-0

Siliciano, R. F., Pratt, J. C., Schmidt, R. E., Ritz, J., and Reinherz, E. L. (1985). Activation of cytolytic T lymphocyte and natural killer cell function through the T11 sheep erythrocyte binding protein. *Nature* 317, 428–430. doi: 10.1038/317428a0

Sinclair, J. (2010). Chromatin structure regulates human cytomegalovirus gene expression during latency, reactivation and lytic infection. *Biochim. Biophys. Acta* 1799, 286–295. doi: 10.1016/j.bbagrm.2009.08.001

Sinclair, J., and Reeves, M. (2014). The intimate relationship between human cytomegalovirus and the dendritic cell lineage. *Front. Microbiol.* 5:389. doi: 10.3389/fmicb.2014.00389

Sinzger, C., Digel, M., and Jahn, G. (2008). Cytomegalovirus cell tropism. *Curr. Top. Microbiol. Immunol.* 325, 63–83. doi: 10.1007/978-3-540-77349-8_4

Sloan, E., Orr, A., and Everett, R. D. (2016). MORC3, a component of PML nuclear bodies, has a role in restricting herpes simplex virus 1 and human cytomegalovirus. *J. Virol.* 90, 8621–8633. doi: 10.1128/JVI.00621-16

Slobedman, B., Barry, P. A., Spencer, J. V., Avdic, S., and Abendroth, A. (2009). Virus-encoded homologs of cellular interleukin-10 and their control of host immune function. *J. Virol.* 83, 9618–9629. doi: 10.1128/JVI.01098-09

Smith, M. S., Bentz, G. L., Alexander, J. S., and Yurochko, A. D. (2004). Human cytomegalovirus induces monocyte differentiation and migration as a strategy for dissemination and persistence. *J. Virol.* 78, 4444–4453. doi: 10.1128/jvi.78.9.4444-4453.2004

Smith, W., Tomasec, P., Aicheler, R., Loewendorf, A., Nemèovièová, I., Wang, E. C. Y., et al. (2013). Human cytomegalovirus glycoprotein UL141 targets the TRAIL death receptors to thwart host innate antiviral defenses. *Cell Host Microbe* 13, 324–335. doi: 10.1016/j.chom.2013.02.003

Söderberg-Nauclér, C., Fish, K. N., and Nelson, J. A. (1997). Interferon-gamma and tumor necrosis factor-alpha specifically induce formation of cytomegalovirus-permissive monocyte-derived macrophages that are refractory to the antiviral activity of these cytokines. *J. Clin. Invest.* 100, 3154–3163. doi: 10.1172/JCI119871

Song, J., Lee, S., Cho, D. Y., Lee, S., Kim, H., Yu, N., et al. (2019). Human cytomegalovirus induces and exploits Roquin to counteract the IRF1-mediated antiviral state. *Proc. Natl. Acad. Sci. U.S.A.* 116, 18619–18628. doi: 10.1073/pnas.1909314116

Spencer, J. V. (2007). The cytomegalovirus homolog of interleukin-10 requires phosphatidylinositol 3-kinase activity for inhibition of cytokine synthesis in monocytes. *J. Virol.* 81, 2083–2086. doi: 10.1128/JVI.01655-06

Staeheli, P., and Haller, O. (2018). Human MX2/MxB: a potent interferon-induced postentry inhibitor of herpesviruses and HIV-1. *J. Virol.* 92:e00709-18. doi: 10.1128/JVI.00709-18

Stahl, S., Burkhart, J. M., Hinte, F., Tirosh, B., Mohr, H., Zahedi, R. P., et al. (2013). Cytomegalovirus downregulates IRE1 to repress the unfolded protein response. *PLoS Pathog.* 9:e1003544. doi: 10.1371/journal.ppat.1003544

Steinle, A., Li, P., Morris, D. L., Groh, V., Lanier, L. L., Strong, R. K., et al. (2001). Interactions of human NKG2D with its ligands MICA, MICB, and homologs of the mouse RAE-1 protein family. *Immunogenetics* 53, 279–287. doi: 10.1007/s002510100325

Stempel, M., Chan, B., and Brinkmann, M. M. (2019). Coevolution pays off: herpesviruses have the license to escape the DNA sensing pathway. *Med. Microbiol. Immunol.* 208, 495-512. doi: 10.1007/s00430-019-00582-0

Stern-Ginossar, N., Weisburd, B., Michalski, A., Le, V. T. K., Hein, M. Y., Huang, S.-X., et al. (2012). Decoding human cytomegalovirus. *Science* 338, 1088–1093. doi: 10.1126/science.1227919

Tahara-Hanaoka, S., Shibuya, K., Onoda, Y., Zhang, H., Yamazaki, S., Miyamoto, A., et al. (2004). Functional characterization of DNAM-1 (CD226) interaction with its ligands PVR (CD155) and nectin-2 (PRR-2/CD112). *Int. Immunol.* 16, 533–538. doi: 10.1093/intimm/dxh059

Tavalai, N., Papior, P., Rechter, S., and Stamminger, T. (2008). Nuclear domain 10 components promyelocytic leukemia protein and hDaxx independently contribute to an intrinsic antiviral defense against human cytomegalovirus infection. *J. Virol.* 82, 126–137. doi: 10.1128/JVI.01685-07

Taylor, R. T., and Bresnahan, W. A. (2006). Human cytomegalovirus immediate-early 2 protein IE86 blocks virus-induced chemokine expression. *J. Virol.* 80, 920–928. doi: 10.1128/JVI.80.2.920-928.2006

Terhune, S., Torigoi, E., Moorman, N., Silva, M., Qian, Z., Shenk, T., et al. (2007). Human cytomegalovirus UL38 protein blocks apoptosis. *J. Virol.* 81, 3109–3123. doi: 10.1128/JVI.02124-06

Tomasec, P., Wang, E. C. Y., Davison, A. J., Vojtesek, B., Armstrong, M., Griffin, C., et al. (2005). Downregulation of natural killer cell-activating ligand CD155 by human cytomegalovirus UL141. *Nat. Immunol.* 6, 181–188. doi: 10.1038/ni1156

Tu, W., and Rao, S. (2016). Mechanisms underlying T cell immunosenescence: aging and cytomegalovirus infection. *Front. Microbiol.* 7:2111. doi: 10.3389/fmicb.2016.02111

Ulbrecht, M., Martinozzi, S., Grzeschik, M., Hengel, H., Ellwart, J. W., Pla, M., et al. (2000). Cutting edge: the human cytomegalovirus UL40 gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis. *J. Immunol.* 164, 5019–5022. doi: 10.4049/jimmunol.164.10.5019

Varani, S., Frascaroli, G., Homman-Loudiyi, M., Feld, S., Landini, M. P., and Söderberg-Nauclér, C. (2005). Human cytomegalovirus inhibits the migration of immature dendritic cells by down-regulating cell-surface CCR1 and CCR5. *J. Leukoc. Biol.* 77, 219–228. doi: 10.1189/jlb.0504301

Viswanathan, K., Smith, M. S., Malouli, D., Mansouri, M., Nelson, J. A., and Früh, K. (2011). BST2/Tetherin enhances entry of human cytomegalovirus. *PLoS Pathog.* 7:e1002332. doi: 10.1371/journal.ppat.1002332

Wagenknecht, N., Reuter, N., Scherer, M., Reichel, A., Müller, R., and Stamminger, T. (2015). Contribution of the major ND10 proteins PML, hDaxx and Sp100 to the regulation of human cytomegalovirus latency and lytic replication in the monocytic cell line THP-1. *Viruses* 7, 2884–2907. doi: 10.3390/v7062751

Wagner, C. S., Walther-Jallow, L., Buentke, E., Ljunggren, H.-G., Achour, A., and Chambers, B. J. (2008). Human cytomegalovirus-derived protein UL18 alters the phenotype and function of monocyte-derived dendritic cells. *J. Leukoc. Biol.* 83, 56–63. doi: 10.1189/jlb.0307181

Waldman, W. J., Knight, D. A., Huang, E. H., and Sedmak, D. D. (1995). Bidirectional transmission of infectious cytomegalovirus between monocytes and vascular endothelial cells: an in vitro model. *J. Infect. Dis.* 171, 263–272. doi: 10.1093/infdis/171.2.263

Wang, E. C. Y., McSharry, B., Retiere, C., Tomasec, P., Williams, S., Borysiewicz, L. K., et al. (2002). UL40-mediated NK evasion during productive infection with human cytomegalovirus. *Proc. Natl. Acad. Sci. U.S.A.* 99, 7570–7575. doi: 10.1073/pnas.112680099

Wang, E. C. Y., Pjechova, M., Nightingale, K., Vlahava, V.-M., Patel, M., Ruckova, E., et al. (2018). Suppression of costimulation by human cytomegalovirus promotes evasion of cellular immune defenses. *Proc. Natl. Acad. Sci. U.S.A.* 115, 4998–5003. doi: 10.1073/pnas.1720950115

Wang, N., Baldi, P. F., and Gaut, B. S. (2007). Phylogenetic analysis, genome evolution and the rate of gene gain in the Herpesviridae. *Mol. Phylogenet. Evol.* 43, 1066–1075. doi: 10.1016/j.ympev.2006.11.019

Warren, C. J., Griffin, L. M., Little, A. S., Huang, I.-C., Farzan, M., and Pyeon, D. (2014). The antiviral restriction factors IFITM1, 2 and 3 do not inhibit infection of human papillomavirus, cytomegalovirus and adenovirus. *PLoS One* 9:e96579. doi: 10.1371/journal.pone.0096579

Weisblum, Y., Oiknine-Djian, E., Zakay-Rones, Z., Vorontsov, O., Haimov-Kochman, R., Nevo, Y., et al. (2017). APOBEC3A is upregulated by human cytomegalovirus (HCMV) in the maternal-fetal interface, acting as an innate anti-HCMV effector. *J. Virol.* 91:e01296-17. doi: 10.1128/JVI.01296-17

Wilkinson, G. W., Kelly, C., Sinclair, J. H., and Rickards, C. (1998). Disruption of PML-associated nuclear bodies mediated by the human cytomegalovirus major immediate early gene product. *J. Gen. Virol.* 79(Pt 5), 1233–1245. doi: 10.1099/0022-1317-79-5-1233

Woodhall, D. L., Groves, I. J., Reeves, M. B., Wilkinson, G., and Sinclair, J. H. (2006). Human Daxx-mediated repression of human cytomegalovirus gene expression correlates with a repressive chromatin structure around the major immediate early promoter. *J. Biol. Chem.* 281, 37652–37660. doi: 10.1074/jbc.M604273200

Wu, J., Chalupny, N. J., Manley, T. J., Riddell, S. R., Cosman, D., and Spies, T. (2003). Intracellular retention of the MHC class I-related chain B ligand of NKG2D by the human cytomegalovirus UL16 glycoprotein. *J. Immunol.* 170, 4196–4200. doi: 10.4049/jimmunol.170.8.4196

Xiaofei, E., and Kowalik, T. F. (2014). The DNA damage response induced by infection with human cytomegalovirus and other viruses. *Viruses* 6, 2155–2185. doi: 10.3390/v6052155

Xie, M., Xuan, B., Shan, J., Pan, D., Sun, Y., Shan, Z., et al. (2015). Human cytomegalovirus exploits interferon-induced transmembrane proteins to facilitate morphogenesis of the virion assembly compartment. *J. Virol.* 89, 3049–3061. doi: 10.1128/JVI.03416-14

Xu, Y., Ahn, J. H., Cheng, M., Aprhys, C. M., Chiou, C. J., Zong, J., et al. (2001). Proteasome-independent disruption of PML oncogenic domains (PODs), but not covalent modification by SUMO-1, is required for human cytomegalovirus immediate-early protein IE1 to inhibit PML-mediated transcriptional repression. *J. Virol.* 75, 10683–10695. doi: 10.1128/JVI.75.22.10683-10695.2001

Xuan, B., Qian, Z., Torigoi, E., and Yu, D. (2009). Human cytomegalovirus protein pUL38 induces ATF4 expression, inhibits persistent JNK phosphorylation, and suppresses endoplasmic reticulum stress-induced cell death. *J. Virol.* 83, 3463–3474. doi: 10.1128/JVI.02307-08

Yan, N., and Chen, Z. J. (2012). Intrinsic antiviral immunity. *Nat. Immunol.* 13, 214–222. doi: 10.1038/ni.2229

Zhang, K., and van Drunen Littel-van den Hurk, S. (2017). Herpesvirus tegument and immediate early proteins are pioneers in the battle between viral infection and nuclear domain 10-related host defense. *Virus Res.* 238, 40–48. doi: 10.1016/j.virusres.2017.05.023

Zhao, R., Hu, M., Liang, S., Wang, B., Yu, B., Yang, G., et al. (2019). IE86 inhibits the apoptosis and promotes the cell proliferation of glioma cells via the hnRNP A2/B1-mediated alternative splicing of Bcl-x. *Int J Clin Exp Pathol.* 12, 2775–2785.

Zheng, Q., Tao, R., Gao, H., Xu, J., Shang, S., and Zhao, N. (2012). HCMV-encoded UL128 enhances TNF-α and IL-6 expression and promotes PBMC proliferation through the MAPK/ERK pathway in vitro. *Viral Immunol.* 25, 98–105. doi: 10.1089/vim.2011.0064

Zingoni, A., Molfetta, R., Fionda, C., Soriani, A., Paolini, R., Cippitelli, M., et al. (2018). NKG2D and its ligands: "One for All, All for One.". *Front. Immunol.* 9:476. doi: 10.3389/fimmu.2018.00476

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Dell'Oste, Biolatti, Galitska, Griffante, Gugliesi, Pasquero, Zingoni, Cerboni and De Andrea. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.